

**Salivary Cortisol as a Measure of Pain and Distress in  
Sick and Preterm Infants.**

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## **DECLARATION**

This thesis is entirely my own composition and has not been accepted or submitted for any other degree or professional qualification. The work that is described was performed by myself, except where specifically quoted in the text.



## Abstract

### Salivary cortisol as a measure of pain and distress in sick and preterm infants

**Background** Untreated pain and distress in the newborn infant, particularly the preterm infant, can affect clinical outcome and have long-term effects on neurological development. There is currently no reliable tool for assessing sustained pain or distress in sick and preterm newborn infants. Salivary cortisol has been used as a tool to assess stress in older infants, children and adults, but not this population.

**Objective** To evaluate salivary cortisol as a potential tool for assessing sustained distress or pain in sick and preterm infants.

#### Design

- 1) Four different saliva collection methods were evaluated for their ease of application in the population and their effect on cortisol radioimmunoassay.
- 2) 50 matched saliva-plasma samples were taken in order to explore the relationship between salivary and total plasma cortisol and to estimate the range of values in the study population.
- 3) A longitudinal study of weekly, morning and evening salivary cortisol was undertaken in 11 infants to evaluate the nature of potential circadian rhythm in cortisol secretion.
- 4) Saliva samples were taken from infants in 4 'presumed distress' groups:
  - i) Controls (n=40)
  - ii) Infants requiring continuous positive airways pressure (CPAP) (n=10)
  - iii) Infants requiring conventional mechanical ventilation (n=10)
  - iv) Infants with meningitis or necrotising enterocolitis (n=10)

Levels from the four groups were compared along with illness severity scores.

Abstract cont.

**Subjects** All subjects were in-patients on a neonatal unit in a tertiary referral centre at a teaching hospital.

**Measurements** Cortisol was measured in saliva in 100 µl samples by highly specific radioimmunoassay without prior extraction.

## Results

- 1) Citric acid as a potential sialogogue was found to be distressing to the infants, and cotton and polyester swabs resulted in cortisol values significantly higher and more variable than 'plain' saliva ( $p=0.01$ , paired t-test).
- 2) Total plasma and salivary cortisol were significantly related using Spearman's correlation;  $r = 0.75$ ,  $p < 0.01$

Total plasma cortisol; median 244.2 nmol L<sup>-1</sup>, range 37.3-3802 nmol L<sup>-1</sup>, interquartile range 94.1-418.0 nmol L<sup>-1</sup>

Salivary cortisol; median 43.1 nmol L<sup>-1</sup>, range 2.8-3767 nmol L<sup>-1</sup> interquartile range 14.0-121.3 nmol L<sup>-1</sup>

No infant born < 30 weeks gestation, followed till discharge home, had a period of 4 or more weeks of sustained morning > evening salivary cortisol.

- 3) Salivary cortisol levels in infants in 'presumed distress' groups were significantly higher than in control groups ( $p < 0.01$ ). Illness severity assessed by SNAP and CRIB scores did not contribute significantly to salivary cortisol levels (General linear model,  $p>0.05$ ). No difference was found for comparisons between salivary cortisol in infants who were term and preterm, previously sick and previously well, and across postnatal age groups.

## **Conclusions**

1. Salivary cortisol may be used as a practical tool to assess adrenocortical activity in sick and preterm infants. Direct suction of 'plain' saliva from an infant's mouth, without citric acid, was chosen as the most suitable collection method. The effect of cotton and polyester swabs was presumed to be due to cross-reactivity of swab constituents with the assay antibody.
2. Salivary levels correlated well with total plasma cortisol levels and were higher than previously reported for any population.
3. Classical circadian rhythm in cortisol secretion is not apparent in very preterm infants prior to discharge from hospital.
4. Infants with presumably distressing conditions and treatments had significantly higher salivary cortisol than controls, and illness severity was not as strongly associated with salivary cortisol levels as the degree of presumed distress. Salivary cortisol levels in 'well' infants do not vary significantly over the first weeks of life, are not significantly different in preterm and term infants at the age of 3 postnatal days, and are not affected by previous illness.

## DEDICATION

This thesis is dedicated to my family;

Stuart, my husband, for all his loving and patient support and encouragement,  
and for taking the boys for adventures while I worked.

Ewan and Lewis, my sons, who tolerated my absence and distraction while I  
worked, and cheered me immeasurably when I stopped.

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List of abbreviations

Abbreviation	Full text
11 $\beta$ HSD	11 $\beta$ hydroxysteroid dehydrogenase
17OHP	17 hydroxyprogesterone
3 $\beta$ HSD	3 $\beta$ Hydroxysteroid dehydrogenase
ACTH	Adrenocorticotrophic Hormone
AGA	Appropriate for gestational age
AVP	Arginine Vasopressin
BP	Blood Pressure
BW	Birth weight
BPD	Bronchopulmonary dysplasia
CBG	Cortisol binding globulin
CLD	Chronic lung disease
CPAP	Continuous positive airways pressure
CRH	Corticotrophin releasing hormone
CRIB	Clinical risk index for babies
CSF	Cerebrospinal fluid
DBPCT	Double blind placebo controlled trial
dex	Dexamethasone
DHEA	Dehydroepiandrosterone
DHEAS	Dehydroepiandrosterone-sulphate

E	Cortisone
ELBW	Extremely low birth weight (<1000g)
F	Cortisol
GA	Gestational age
GLM	General linear model
HFOV	High frequency oscillatory ventilation
HPA	Hypothalamo-pituitary-adrenal
IASP	International association for the study of pain
IFA	Immunofluorescent assay
IL-1	Interleukin-1
IL-6	Interleukin-6
IPPV	Intermittent positive pressure ventilation
IUGR	Intrauterine growth retardation
IVH	Intraventricular haemorrhage
LBW	Low birth weight (<2500g)
LP	Lumbar puncture
MS	Mass Spectrometry
mRNA	Messenger Ribonucleic acid
NBAS	Neonatal Brazelton assessment scale
NEC	Necrotising enterocolitis
PBM	Protein binding method of Murphy

PCA	Post conceptional age
PDA	Patent ductus arteriosus
PNA	Post natal age
PVL	Periventricular leukomalacia
RDS	Respiratory distress syndrome
RIA	Radioimmunoassay
SCN	Suprachiasmatic nuclei
SD	Standard deviation
SNAP	Score for neonatal acute physiology
SNRP	Stress non responsive period
SVD	Spontaneous vaginal delivery
TNF $\alpha$	Tissue necrosis factor $\alpha$
TPN	Total parenteral nutrition
VLBW	Very low birth weight (<1500g)

## **1 Project Outline**

It is now widely accepted that pain and distress exist in newborn infants, both term and preterm. The effects of such pain and distress on an infant's clinical condition can range from seconds to days. Longer-term effects on neurological development potentially extend to weeks, months and years. There is evidence that appropriate treatment of pain and distress may be advantageous to sick infants. These issues and the relevant literature are discussed in chapter 2.1.

With such a body of evidence available, it would be advantageous to develop a reliable tool to measure pain and distress. Only then, will it be possible to detect the problem and monitor the success of its management. There are behavioural and simple physiological tools for the assessment of acute pain or distress, but little attention has been paid to the assessment or measurement of sustained distress. It is unlikely that behavioural assessment alone will be adequate in this situation. The literature on current pain and distress scores is discussed in chapter 2.2.

Salivary cortisol is now widely accepted as a reliable indicator of adrenocortical activity in healthy term infants, children and adults (chapter 2.4). In these populations, cortisol levels are closely associated with negative affect, pain and distress. Salivary cortisol might thus mirror distress in preterm and sick newborn infants.

It is relatively simple to ask an adult or school-aged child to spit saliva into a collection pot but this is impractical in infants. Potential collection methods for use in sick and preterm infants were therefore evaluated (chapter 4). The selection and development of a suitable collection method is described.

The relationship between salivary and plasma cortisol in term infants, children and adults has been well described, but the physiology of the preterm and sick infants' salivary glands,

cortisol metabolism and cortisol regulation may be different from the more mature infant and adult. This difference may be so marked that salivary cortisol is not a reliable indicator of adrenocortical activity in such infants. To address this issue, the relationship between plasma and salivary cortisol in sick and preterm infants is explored (chapter 5).

Circadian rhythm is a feature of a healthy hypothalomo-adrenocortical system in the older infant, child and adult. Term infants develop circadian rhythm in cortisol levels at about three months postnatal age and it has been suggested that preterm infants might develop circadian rhythms at earlier post menstrual age than term infants. In order to time sampling and interpret results, the presence of a classical circadian rhythm in cortisol levels in infants born <30 weeks GA was sought (chapter 6).

There is no gold standard for pain or distress measurement in sick or preterm infants. It was therefore assumed that conditions or procedures that adults would find distressing would also be perceived as distressing to the infants in the study. Infants were grouped by procedure and condition into 'presumed distress' groups and their salivary cortisol levels were compared to each other and controls (chapter 8).

It is possible that sicker infants will be in more pain or require more distressing interventions than less sick infants. Cortisol may mirror physiological illness severity rather than psychological distress. An assessment of physiological illness severity (chapter 2.6) was therefore made for each infant at birth and at the time of the saliva sampling. This was analysed with the data from 'presumed distress' groups as a potential major confounding factor in determining cortisol levels. The results are discussed in light of the literature on the relationship between illness severity and plasma cortisol in sick and preterm infants (chapter 2.5 and chapter 8).

## **2 Background and review of the literature**

### **2.1 Pain and distress in new-born infants.**

#### **2.1.1 Definitions and terminology**

'Pain' describes the 'conscious perception of nociception'. It is a subjective experience that requires truthful self-report for confirmation of its presence. The International Association for the Study of Pain (IASP) defines pain as an emotional experience based on actual or potential tissue damage or described in terms of such damage (IASP, 1979). The definition also states that pain is subjective and learned through experience in later life (Merskey H, 1991). These definitions are extremely difficult to apply to the preverbal newborn infant. Firstly, newborn infants cannot give the gold standard of self-report, and secondly, they have no previous experience upon which to base their pain perception. Problems with definition also extend to philosophical considerations, specifically the nature and emergence of 'consciousness' in the infant.

In infants, it can only be inferred with a high degree of probability that pain is present. This has led to attempts to define and detect 'apparent probable pain patterns' (Merskey H, 1996). Most of these have been behavioural measures or patterns, and some have included the physiological dimension (Table 2.1). Anand and Craig also addressed the above issues by broadening the definition of pain to include the physiological and behavioural indicators of pain perception. They put the case that pain occurs early in development and is an inherent quality of life that serves as a signalling system for tissue damage (Anand and Craig, 1996). Although most pain scores have been developed and used in the research setting, few have included the neuroendocrine dimension, with variables such as adrenaline and cortisol. None have included cortisol as part of the 'apparent probable pain pattern' of sustained or continuing pain or distress. This is the basis of the present study.

### 2.1.2 'Pain equivalent experience'

An identical pain experience to that perceived by an adult will not exist in a newborn infant simply because the newborn infant's neurology and consciousness are different from those of the adult. It seems logical to accept that a 'pain-equivalent experience' exists in the following circumstances:

1. The existence of 'probable pain patterns' (Merskey H, 1996) of
  - a. behaviour
  - b. physiology
  - c. neuroendocrinology
  - d. metabolism
2. The occurrence of these patterns in circumstances that would evoke pain in the adult  
i.e. we can infer pain from the circumstances.
3. Therapy that has the primary effect of alleviating pain returns the infant's behaviour physiology, neuroendocrinology and metabolism to a more 'normal/ pain-free' pattern.

### 2.1.3 'Distress'

In a medically sick patient, at some ill-defined point, the 'stress' of illness and therapy may become 'distress'. The word, 'distress' used in a situation where pain is highly likely describes associated fear, anxiety, and agitation. Results of a survey of Australian nurses in 1999 revealed that, except for diagnosis, there were no significant differences between the cues carers used to assess pain and those to assess agitation (Ramelet, 1999). In older children, distress correlates closely with pain, but may have other causes. It may be just as important to assess and treat distress, as distress results in physiological instability, reduces



coping mechanisms, and potentiates the perception and effects of pain (Franck *et al.*, 2000). In an older child or adult, pain usually results in distress. 'Pain', 'stress' and 'distress' all describe states that ultimately probably result in suffering and which may have detrimental effects on the well-being of a sick infant (Anand, 1997; Anand, 1998; Anand and Hickey, 1987; Anand, 1993). The boundaries between these states merge. 'Distress' is a term that encompasses the negative sensations and effects of pain and stress in the sick newborn infant. It is used for the purposes of discussion in this project.

#### 2.1.4 Evidence for 'pain' in newborn infants

That both preterm and full-term infants have functioning nociceptive systems was first drawn together in 1987 (Anand and Hickey, 1987).

#### 2.1.5 Transmission of the pain signal

##### a) Neuroanatomy and Neurophysiology of pain 'pathways': adults and older children

1. Transduction and transmission from periphery to dorsal horn of spinal cord
  - a. A-delta fibres: large, myelinated, fast-conducting
  - b. C fibres: small, unmyelinated, slow-conducting
2. Amplification and attenuation of signal by activation of surrounding neurones in the spinal chord and periphery. (eg. Stimulation of A-beta fibres that transmit non-painful touch compete with nociceptive transmission in the dorsal horn of the spinal cord, reducing pain intensity perception.)

Spinal cord transmitters:

- i. Amplification: substance P, calcitonin gene related peptide, neurokinin A

- ii. Attenuation: endogenous opioids, norepinephrine, serotonin, GABA (Gamma amino butyric acid), Glycine
3. Second order neurones: spinothalamic, spinoreticular, spinomesencephalic tracts.
  4. Perception. No single 'pain centre' appears to exist. Perception, emotion, and cognitive meaning occur throughout a 'neuromatrix' (Melzack, 1999).
  5. Descending inhibition: by projections from supra-spinal areas eg. peri-aqueductal grey, raphe nucleus, locus coeruleus. Mediators are: norepinephrine, serotonin, endogenous opioids, GABA and acetyl choline (Stamford, 1995).
  6. Central sensitisation: excitatory amino acids act on NMDA (N-methyl-D-aspartate) receptors producing prolonged depolarisation and 'windup' (Dickenson, 1995).

#### 2.1.6 Presence of 'pain apparatus' from early gestation

'Characteristics of the immature pain system in preterm neonates predisposes them to greater clinical and behavioural sequelae from inadequately treated pain than older age groups' (Anand, 2000a).

The peripheral and central structures required for pain perception are present from between the first and second trimester (Fitzgerald and Anand, 1993). The newborn infant has as many sensory receptors or more than are present in adults. Myelination of ascending pathways (spinal cord, brainstem and thalamus) occurs by 30 weeks, and from thalamus to cortex occurs by 37 weeks. Spinal cord synapses begin developing before 14 weeks and are completed by 30 weeks gestation (Anand and Hickey, 1987). Intra-uterine fetal needling of the abdomen for transfusion results in significant cortisol and endorphin increases at 23-24 weeks (Giannakouloupoulos *et al.*, 1994).

The 'natural' painkiller, enkefalin, is known to be present in low concentrations in the CNS at birth (Rolewicz *et al.*, 1977).

Substance P appears at 12-16 weeks GA, with higher density than adults (Anand and Hickey, 1987).

A developing maturity of the fetal cerebral cortex has been demonstrated by Anand and Hickey (Anand and Hickey, 1987):

1. EEG patterns
2. Cortical evoked potentials elicited before 30 weeks gestation
3. Maximal metabolic rates measured by glucose utilisation in sensory areas of the brain
4. Regular sleep-wake patterns present from 28 weeks gestation
5. Synaptic connection to afferent nerve axons established at 20-24 weeks gestation.

#### 2.1.7 Differences between infants and adults in pain transmission

1. Peripheral pain transmission appears to occur mainly along C fibres (non-myelinated), rather than A-delta (myelinated) fibres.
2. Spinal cord transmission is less precise.
3. Descending inhibitory neurotransmitters are lacking (Fitzgerald, 1991; Ramsay and Lewis, 1995).

#### 2.1.8 Developmental neurological processes

##### 2.1.8.1 Programmed neuronal death

Early in development, there is expansion and surplus of neurones and synapses. Neuronal circuits that are active proliferate, while the inactive will degenerate.

#### 2.1.8.2 *Excitotoxicity*

Excessive depolarisation of neurones results in neuronal dysfunction. Small, inhibitory local circuit neurones are most vulnerable to this process. Thus, excitotoxicity might result in hyperexcitability, and even greater sensitivity to pain (Dubner and Ruda, 1992).

#### 2.1.8.3 *Hyperalgesia*

Tissue injury results in release of inflammatory mediators, eg. potassium, bradykinin, prostaglandins, cytokines, nerve growth factors, catecholamines, substance P. These sensitise A-delta and C fibres and recruit 'silent nociceptors', resulting in hyperalgesia (Dickenson, 1995; Siddall and Cousins, 1995).

#### 2.1.8.4 *Sensitisation*

Infants have low thresholds to von Frey hair testing (Fitzgerald *et al.*, 1988). They may perceive pain more intensely because of their limited ability to modulate transmission (Fitzgerald, 1995; Fitzgerald and Anand, 1993). This appears to result from larger receptive fields, and prolonged responses to nociceptive stimuli (Fitzgerald, 1991). Specific noxious stimuli may thus alter the sensitivity of the neonate's nervous system to other inputs, leaving the infant hyperalgesic (Fitzgerald *et al.*, 1988).

#### 2.1.9 Short term effects of pain.

##### 2.1.9.1 *Post-operative stability*

Untreated pain results in catabolism and hypermetabolism, resulting in hyperkalaemia, increased susceptibility to infection and slowed healing mechanisms. These consequences may ultimately lead to poor outcome and morbidity. This was demonstrated by Anand, who found that adequate analgesia for both preterm and term infants undergoing surgery reduced their stress responses and improved their clinical stability during and after operation (Anand

*et al.*, 1987; Anand *et al.*, 1990.). Regional anaesthetic techniques have also reduced stress response in children <4yrs undergoing surgery (Wolf *et al.*, 1993).

#### 2.1.9.2 IVH

Acute physiological changes caused by painful or stressful stimuli have been implicated as potential factors in the causation or subsequent extension of early intraventricular hemorrhage (IVH) or the ischemic changes leading to periventricular leukomalacia (PVL) (Anand, 1998). However, a large subsequent multi-centre randomised trial has showed no measurable difference in serious brain injury (IVH or PVL) or death in infants treated and those not treated prophylactically with morphine infusions (NEOPAIN study, 2003, currently under submission for publication). The data generated by such trials are complex, and separating the influence that pain and analgesia have on outcome from other factors, as well as the limitations of the currently used outcome measures, make interpretation extremely difficult. There remains no consensus on the use of routine prophylactic infusions of opiates for the treatment of pain and distress (out with the post-operative period) in newborn infants.

#### 2.1.9.3 Routine treatment procedures

In a randomised placebo-controlled trial of morphine vs saline, physiological, plasma beta-endorphin, cortisol, and glucose responses to routine treatment procedures were studied in 84 mechanically ventilated distressed neonates. The duration of hypoxemia and/or arterial blood oxygen saturation during treatment procedures was significantly longer in the saline group and distress was much higher. This study suggests that newborns with respiratory difficulties often suffer from hypoxemia during essential treatment procedures and that the use of opioid analgesia may reduce the duration of hypoxemia and the associated distress and, therefore, may improve the long-term results of neonatal intensive care (Pokela, 1994a).

#### 2.1.9.4 Autonomic resetting and dampened behavioural pain response

Grunau et al described medium term effects on pain response of early exposure to invasive events. The most significant factors associated with altered behavioural and autonomic pain reactivity to blood taking at 32 weeks' postconceptional age were a greater number of previous invasive procedures since birth, and gestational age at birth, both of which were related to a dampened response. The results of this study suggested that the judicious use of analgesia may ameliorate these effects on later pain reactivity, as exposure to morphine seemed to normalise the response. In addition, higher mean heart rate at baseline was associated with lower gestational age at birth and longer time on mechanical ventilation, leading to the suggestion that early pain exposure at very low gestational age may alter the autonomic substrate, resulting in infants who are in a perpetual state of stress (Grunau *et al.*, 2001).

Preterm infants who spend postmenstrual weeks 28 to 32 in neonatal intensive care are less mature in their pain response than newborn premature infants of 32 weeks' postmenstrual age. Greater frequency of invasive procedures is associated with behavioural immaturity, whereas birth factors are associated with physiological immaturity (Johnston and Stevens, 1996).

#### 2.1.10 Medium term effects of pain

The infant's stress response, measured by salivary cortisol, and crying duration after immunisation at 8 weeks was found to be related to mode of delivery, with the greatest response shown in those born by assisted delivery and the least response in those born by elective caesarean section (Taylor *et al.*, 2000).

## 2.1.11 Long term effects of pain

### 2.1.11.1 Pain responses

Boys who have undergone circumcision without analgesia show increased pain reactivity (measured by behaviour scores and crying duration) to the pain of immunisation at 4 and 6 months than those who have not been circumcised (Taddio *et al.*, 1995).

Subsequent research, however, has raised the question of whether differences in later pain responses are significant and whether they represent a long-term effect of early pain experience or a developmental lag in pain response (Oberlander *et al.*, 2000). These authors detected only subtle bio-behavioural differences in pain response to heel-stick between pain and non-pain exposed infants at 4 months corrected age.

Children's judgements about pain at age 8-10 years have been compared between two groups of children with different exposure to nociceptive procedures in the neonatal period; children born with extremely low birthweight ( $\leq 1000$  g,  $n = 47$ ) rated medical pain intensity significantly higher than psychosocial pain, unlike children with full birthweight ( $\geq 2500$  g,  $n = 37$ ). Increased pain affect ratings in recreational and daily living settings were also related to duration of neonatal intensive care unit stay for the extremely low birth weight children (Grunau *et al.*, 1998a).

### 2.1.11.2 Social, emotional, educational etc.

Exposure to repetitive neonatal pain may cause permanent or long-term changes because of the developmental plasticity of the immature brain. Decreased pain thresholds and altered gene expression in the somatosensory cortex were noted in adult rats that were exposed to repetitive pain in the neonatal period when compared to those that were not. The same group of rats, as adults, showed an increased

preference for alcohol; increased latency in exploratory and defensive withdrawal behaviour; and a prolonged chemosensory memory in a social discrimination test. It has been hypothesised that the increased plasticity of the neonatal brain may allow these and other changes in brain development to increase vulnerability to stress disorders and anxiety-mediated adult behaviour (Anand *et al.*, 1999; Anand, 2000b; Anand and Scalzo, 2000).

In a prospective study of 36 children who were extremely low birthweight (<1000 g) preterm infants and 36 matched full-term controls, differences were found in somatisation at age 4.5 years. Only children who had been extremely premature, and thereby experienced prolonged hospitalisation and repeated medical intervention in infancy, had clinically high somatisation scores on the Personality Inventory for Children (Grunau *et al.*, 1994).

One hundred and thirty-seven very low birthweight children were compared at 12 years with a sample of matched peers. The very low birth weight children were found to be more likely to have generalised anxiety, more symptoms of depression and greater than 25% showed a psychiatric disorder of some type, especially ADHD, compared to 9% of peers (Botting *et al.*, 1997).

Early pain experience has also been implicated in educational, psychological, behavioural and emotional difficulties in school aged children and adolescents (Hack *et al.*, 1994; Botting *et al.*, 1997). It is, however, extremely difficult to assess the affect of pain specifically on these outcomes as there are multiple factors at play.



## 2.1.12 The need for reliable measures of sustained pain.

Accurate pain assessment is essential for effective pain management. Evaluation of therapy demands specific, sensitive, reliable measures of pain that can be used in both the research and clinical setting.

### 2.1.12.1 *Prevalence of neonatal pain*

The improvement in the survival of premature infants, particularly those weighing less than 1000g at birth, means that there are now a significant number of infants, many of whom require surgery, undergoing frequent and sometimes extensive traumatic procedures in intensive care units (Barker and Rutter, 1995).

### 2.1.12.2 *Why sustained pain is different from acute pain*

A model has been suggested in which acute and sustained pain responses parallel the infant's response to maternal separation. Thus, acute pain precipitates the communication of distress, with energy expenditure on body movements, crying, and changes in facial expression. There are 'fight or flight' type changes in physiology. With the continuation of pain, decreased body movements, expressionless face, and passivity predominate. Decreased heart rate, respiratory variability and O<sub>2</sub> consumption suggest a 'shut down' of energy expenditure (Anand, 1997). While most pain assessment tools are designed to detect and quantify pain due to acute, isolated, procedural pain, most of these infants are also exposed to sustained and repeated painful or distressing events. The scores have often been developed on groups of relatively well and relatively mature infants. There are few measures to assess chronic pain in infants, and even fewer measures for infants who are low birth weight, critically ill, or ventilated (Abu-Saad *et al.*, 1998; Larsson, 1999; Whitfield and Grunau, 2000).

### 2.1.12.3 Lack of effective pain management in current clinical practice

A recent study in Sweden showed that few neonatal units attempted to assess pain. Documentation of pain intensity was 'inadequate' and 'should be improved' (Gradin, 2000).

A study of the factors influencing individuals making pain assessments in newborn infants found that there was a tendency to judge early preterm infants to be experiencing less pain than term infants even though they were subjected to the same invasive procedure as the more mature infants (Hadjistavropoulos *et al.*, 1997).

A survey in 1997 showed that postoperative pain in neonates in Canadian NICUs appears to be consistently treated, primarily with opioid analgesics, but analgesia, opioid or nonopioid, is rarely given for non-surgical invasive procedures (Johnston *et al.*, 1997a)

### 2.1.13 Neuroendocrinology and pain in the new-born

The work of Anand that 'set the ball rolling' in the field of neonatal pain research and the neuroendocrinology associated with medical and surgical pain/stress/distress in the newborn infant is discussed in detail in chapter 2.3.

### 2.1.14 Summary

Despite difficulties with nomenclature, there is ample evidence that newborn infants, including those born prematurely, possess the means to experience pain and distress, exhibit their effects, benefit from their management in certain situations, and may be adversely affected if pain or distress are untreated. Sustained or continuing pain is a relevant and common entity that is at presently poorly understood, assessed and treated.

## **2.2 Assessment of pain and distress in the newborn**

Table 2.1 (page 52) shows the currently available tools for pain or distress assessment in the newborn.

### **2.2.1 Describing vs detecting pain**

Most pain assessment tools are based on descriptions of behavioural and physiological pain reactions to, for example, heel lancing for blood collection. Thus the majority of observational and descriptive pain research is based on pain of known time, source, nature, site, intensity and context. An ideal pain assessment tool would allow clinicians to be alerted to the presence of pain in the 'real' situation, when the onset, nature, source, intensity, etc of pain are not always obvious. Stevens *et al* also point out that while 'defining' the premature infant's behavioural response to pain assists in clarifying the premature infant's reaction to a tissue damaging stimulus, it does not always guarantee correct 'identification' of pain in individual infants (Stevens *et al.*, 1994).

The considerable overlap of magnitude of physiological and behavioural pain response with invasiveness of procedure suggests that there is not a physiological or behavioural threshold that clearly marks the presence of pain. Inconsistencies in physiological and behavioural responses make reliance on a pain index difficult. It has been argued that as we have no adequate pain assessment tool, the best approach may be one of prophylactic provision of systematic pain management to reduce the acute and long-term impact of early procedural pain (Porter *et al.*, 1999).

### **2.2.2 Acute vs continuing pain**

There is one tool that has been proposed for clinical assessment of prolonged pain in the preterm infant; the EDIN score (table 2.1) (Debillon *et al.*, 2001). While this looks promising, it is not yet in widespread use and has not been extensively appraised. Several of the parameters used such as 'quality of contact with nurses' and 'consolability' are by their

nature subjective, so constituting a potential drawback. The scoring system thus presumes substantial experience and skill on the part of the nurse. Also, no account has been taken of gestational age, behavioural state, previous pain experience, or illness severity in the development or application of the score. The authors did not discuss ventilation and the resultant problems interpreting facial and other behavioural parameters. It is however in the early stages of development.

As described in chapter 2.1, a model has been suggested in which acute and sustained pain responses parallel the infant's response to maternal separation. Applying this model, sustained pain would be associated with decreased body movements, expressionless face, and passivity (Anand, 1997). The EDIN assessment tool is the only one described that takes into consideration these potential manifestations of pain or distress.

### 2.2.3 Clinical application of assessment tools

The CRIES, NIPS, PIPP and NFCS have been shown to be reliable and effective in the clinical situation. The other tools are research orientated or require further validation in the clinical setting.

### 2.2.4 Facial expression

Facial expression has been one of the most studied indicators of pain, and is accepted as one of the most consistent measures of acute pain for different age groups and situations (Grunau *et al.*, 1998b; Grunau *et al.*, 1990; Grunau and Craig, 1987). The cluster of facial activity associated with pain is similar in adults and newborns, both full-term and preterm, providing construct validity for the proposition that the face 'encodes' painful distress in infants and adults (Craig *et al.*, 1994).

### 2.2.5 Body movements

The significance of body movements is still poorly understood (Craig *et al.*, 1984). A recent study found that some extensor movements seemed to be distress signals, whereas tremors,

startles, and twitches were not related to discomfort during procedures likely to cause distress. The point was made that these behaviours may differ qualitatively during longer lasting tissue invasive events, indicating the need for more in-depth study of patterns of motor activity in preterm infants over longer observation periods to evaluate potential signs of stress and pain in babies undergoing intensive medical care (Grunau *et al.*, 2000).

#### 2.2.6 Cry

Some researchers have suggested that crying can be used to measure pain in newborn infants only when the cause of crying is known (Runefors *et al.*, 2000). This is due to the large differences between individual cries from a single infant, as well as in the duration of each cry, total crying time, and fundamental frequencies between infants (Fuller, 1991; Porter *et al.*, 1986).

One study examining the effect of various comfort measures during heel lance found that there were proportionately fewer cries in two pacifier groups compared to prone positioning and standard care groups, and cry duration was positively correlated with PIPP scores. However, neither cry duration nor fundamental frequency reflected group differences. Further research is needed to determine if cry is a sensitive and valid indicator of pain in preterm infants (Johnston *et al.*, 1999a).

Essentially, the problem with 'cry' is that crying is not unique to pain, and pain does not always illicit cry.

#### 2.2.7 Multidimensional assessment: relationship between behaviour and physiology

It has been shown that various comfort giving measures can reduce the behavioural response to pain (Gormally *et al.*, 2001; Gray *et al.*, 2000). However, the aims of pain treatment are to decrease the distress precipitated by the pain, and not just the expression of that distress, and to decrease the potentially detrimental effects of the physiological disturbance associated

with the pain. It is therefore important to understand the extent to which pain behaviour reflects pain-associated physiological disruption.

An interesting study has shown that while sucrose and rocking can decrease the behavioural response to pain, heart rate was not decreased by these interventions compared to controls (Johnston *et al.*, 1997b).

In a study designed to examine the relationship between behavioural and physiological indices after major surgery, neonates were found to have lower behaviour-physiology correlations than the older infants, due to low pain scores. Pain characteristics significantly predicted the COMFORT 'behaviour': heart rate and blood pressure correlations, suggesting that the behaviour-physiology correlations increase with increasing pain. There were large inter-individual differences in behaviour-physiology correlations after major surgery (van Dijk *et al.*, 2001).

#### 2.2.8 Factors that affect pain response and measurement

1. Clinical context/ knowledge of assessors
2. Non pharmacological pain reducing measures
3. Sex
4. Race
5. Behavioural state
6. Illness severity
7. Gestational age
8. Postnatal age
9. Pain experience
10. Drugs

#### *2.2.8.1 Clinical context/ knowledge of assessors*

One study examined the effect that clinical background had on pain scores allocated by groups of staff to infants on videotape. The group that read clinical background data prior to scoring gave higher mean levels of assessed pain per videotaped infant than did the group who only viewed the videotapes (Fuller *et al.*, 1999).

#### *2.2.8.2 Non-pharmacological pain reducing measures*

Sweet taste and holding interventions combined in complex ways when acting on different behavioural and physiological response systems to modify expression of distress during heel lance procedure (Gormally *et al.*, 2001). Skin to skin contact has been shown to reduce crying and grimacing by 82% and 65%, respectively, from control infant levels during the heel lance procedure. Heart rate has also been reduced substantially by contact (Gray *et al.*, 2000).

#### *2.2.8.3 Sex*

While most studies of infant pain have found little difference between the sexes in behavioural and physiological pain response, some subtle differences have been noted. Sex differences were apparent in speed of response to heel lance, with boys showing shorter time to cry and to display facial action (Grunau and Craig, 1987). Recently born female neonates of all gestational ages expressed more facial features of pain than male infants during heel lance and 1 minute afterwards (Guinsburg *et al.*, 2000).

#### *2.2.8.4 Race*

Some research has suggested the presence of differences in acute pain response between Chinese and non-Chinese healthy infants by at least 2 months of age (Rosmus *et al.*, 2000)

#### 2.2.8.5 Behavioural state

Awake-alert but inactive infants responded to heel lance with the most facial activity, consistent with current views that infants in this state are most receptive to environmental stimulation. Infants in quiet sleep showed the least facial reaction and the longest latency to cry (Grunau and Craig, 1987). Stevens *et al* also found that facial action in response to heel lance is lessened in infants in deep sleep states (Stevens *et al.*, 1994).

Handling or immobilisation immediately prior to heel lance results in higher physiological and behavioural changes (Sparshott M, 1996). Newborns who are younger, asleep, and have undergone a painful event more recently have been shown to be less likely to demonstrate behavioural and physiologic indicators of pain (Johnston *et al.*, 1999b). Behavioural state was found to influence the physiological responses in response to heel lance in premature infants born between 32 and 34 weeks gestational age (Stevens and Johnston, 1994).

#### 2.2.8.6 Illness severity

Severity of illness modified the acoustic cry variables in premature infants born between 32 and 34 weeks gestational age in response to heel lance (Stevens *et al.*, 1994).

#### 2.2.8.7 Gestational age

In a study of infants of gestational ages 26-31 weeks, Johnston *et al* found that in behavioural and physiological variables, infants of lower gestational age responded less robustly than infants of greater gestational age, but were still capable of responding to acute pain in both behavioural and physiological dimensions (Johnston *et al.*, 1995).

Porter reported similar findings that infants born at <28 weeks' gestation exhibited increased increments in response magnitude with increasing postnatal age (Porter *et al.*, 1999). Neonates with gestational ages as low as 25-27 weeks displayed physiological responsiveness to heel lance, and the heart rate measure was found to vary with gestational age (Craig *et al.*, 1993). Dampened behavioural and autonomic pain reactivity at 32 weeks'



postconceptional age was found in one study to be related to gestational age at birth (Grunau *et al.*, 2001).

Bodily activity in response to heel lance was found to be diminished in preterm neonates in general, relative to full-term newborns (Craig *et al.*, 1993). Premature infants also had shorter, higher-pitched, harsher cries than others in response to acute pain (Johnston *et al.*, 1993; Johnston *et al.*, 1996).

The same facial responses to acute pain were found to be present in preterm infants, but to a lesser extent than term infants (Stevens *et al.*, 1994). Craig *et al.* found that facial activity in response to heel lance increased with the gestational age (25-27 weeks) of the infant. Specificity of the response to the heel lance was greatest on the facial activity measure compared to physiological and body movement measures (Craig *et al.*, 1993).

#### *2.2.8.8 Postnatal age*

In a study of infants born at 28 weeks gestation and followed for 8 weeks, the magnitude of physiological (heart rate and oxygen saturations) and behavioural (three upper facial actions) response to both real and sham heel lance increased with increasing postnatal age. Thus, the older the infant, the more robust and recognisable the response (Johnston *et al.*, 1996).

#### *2.2.8.9 Pain experience*

Newborns who have undergone a painful event recently are less likely to demonstrate behavioural and physiologic indicators of pain (Johnston *et al.*, 1999b). A significant factor associated with dampened behavioural and autonomic pain reactivity at 32 weeks' postconceptional age is a greater number of previous invasive procedures since birth (Grunau *et al.*, 2001).

#### *2.2.8.10 Drugs*

Exogenous steroid exposure has been found to make an independent contribution to both behavioural and autonomic pain scores, in the direction of dampening the response.

Conversely, previous exposure to morphine has been associated with "normalized" (ie, increased rather than diminished) behavioural responses to pain (Grunau *et al.*, 2001).

#### 2.2.9 Causes of distress in the newborn infant receiving intensive care.

##### 1. Ventilation;

Presence of endo-tracheal tube and fixation devices

Distress of mandatory ventilator breathes ('Fighting the ventilator')

Restriction of movement and posture required for ventilation

##### 2. Repeated acute invasive procedures;

Endotracheal suction

Arterial/ venous/capillary blood sampling,

Re-siting of drips

##### 3. Repeated relatively non-invasive procedures;

Transcutaneous gas monitoring probe changes

Bolus naso- or oro-gastric feeds

Drug administration

Blood pressure measurement using inflatable cuffs

##### 4. Minor surgical procedures;

Chest drain insertion

Supra-pubic aspiration of urine

Lumbar puncture

Ventricular tap

5. Co-existing infective/inflammatory conditions;

Necrotising enterocolitis

Osteomyelitis

Meningitis

Generalised sepsis

6. Complications of necessary procedures;

Cellulitis or abscess from infected drip site

Cutaneous probe burns

7. Post operative following major surgery;

Patent ductus arteriosus ligation

Laser therapy for retinopathy of prematurity

Bowel repair/ resection following perforation or necrotising enterocolitis

8. Disruptive handling

Positioning for x-rays

Head ultrasound scans

General care-giving procedures, e.g. nappy changes

9. Environmental stress

Excessive light, either daylight or from photo-therapy

Excessive and distressing sound from monitor alarms, banging bin lids and doors

Unfamiliar tactile environment without containment or fluidity

## 10. Physiological stress

Drug withdrawal

Respiratory insufficiency/ 'air hunger'

Nutritional i.e. hunger

### 2.2.10 Indices used in pain and distress assessment

#### 1. Behaviour

- (a) Cry: spectrographical analysis, latency, duration, and intensity may be used in pain assessment, but these parameters are not applicable to intubated infants. The mere presence of attempted crying despite an endo-tracheal tube indicates significant distress.
- (b) Facial expression alteration with brow bulge, eye squint or squeeze, naso-labial furrowing, mouth or lip purse, tongue tautness and chin quiver.
- (c) Tone exaggeration, with startling or twitching.
- (d) Changes in tone, with general diffuse increase in activity, flexion of trunk and extremities, fetal posturing or arching, leg extension, finger splays or fisting
- (e) Sleep cycle disruption or sleep accompanied by twitches, sounds, jerks, irregular respirations, whimpers or grimaces.
- (f) Observed self regulatory or comforting behaviour such as lowered state, posture changes, hand to mouth movements, sucking, grasping or an expression of 'focused alertness.'

#### 2. Physiological

- (a) Heart rate increase
- (b) Respiratory rate increases, but decrease in immediate acute pain

- (c) Mean arterial pressure increases
- (d)  $PO_2$  varies
- (e) Oxygen saturation decreases
- (f) Skin temp fluctuations
- (g) Skin colour changes
- (h) Galvanic skin response increases
- (i) Palmer sweating (after 37 weeks gestation)
- (j) Cerebral circulation swings
- (k) Intracranial pressure increases
- (l) Gastro-intestinal disturbance with hiccups, gagging, grunting or straining

### 3. Used in research

- (a) Neuro-endocrine markers of stress responsive systems, e.g. cortisol, adrenaline, endorphins.
- (b) Metabolic/ biochemical markers of catabolism, e.g. 3-methyl histidine.
- (c) Computerised analysis of physiological data. e.g. vagal tone decreases.

#### 2.2.11 Specific issues regarding the assessment of pain or distress in the ventilated infant

- (a) Cry not possible due to presence of endo-tracheal tube between vocal chords.
- (b) Facial expression obscured by endo-tracheal tube and fixing device or phototherapy goggles.
- (c) Posture and movements altered by presence of leads, drips and splints.

- (d) Infants usually preterm so at different stages of maturity of response to pain.
- (e) Much of the stress will be subacute and so habituation and withdrawal may occur making behaviour even more difficult to interpret.
- (f) Medical compromise alters or blunts response to distress.
- (g) Arousal state modifies responses
- (h) Environmental modulators e.g. light, noise, temperature, physical containment
- (i) Preterm infants may actually be super-sensitive to pain because of their immature neurology
- (j) Agitation may be due to e.g. respiratory insufficiency or drug withdrawal

Figure 2-1 illustrates some of the above points. In particular, the endotracheal tube and fixation device obscure the mouth, oedema prevents the eyes from opening and obscures expression changes, and splints and the use of paralysing agents prevent body movements.

Figure 2-1 Infant receiving intensive care



Score (ref)	Full name	Acute/Chronic	Term/preterm	Face	Body	Physiol	Notes
EDIN (Debillon <i>et al.</i> , 2001)	Echelle Douleur Inconfort Nouveau-ne, neonatal pain and discomfort scale	Prolonged score over 8 hours	Preterm	Facial activity Relaxed Frown etc Frequent + lasting grimace Cry or blank face	Movements (agitation-frozen) Quality of contact with nurses Consolability	Sleep quality	5 elements Construct validity Inter-rater reliability and internal consistency have been demonstrated Intended to be clinical tool
NFCS (Craig <i>et al.</i> , 1994; Grunau <i>et al.</i> , 1998b; Grunau and Craig, 1987)	Neonatal Facial Coding System	Acute	Term and Preterm	Brow-bulge Eye-squeeze Nasolabial furrow Open lips Stretch mouth Lip purse Tongue taut Chin quiver Cry latency and duration	None	Used in conjunction with heart rate changes, sleep/wake state and hand movements.	Awake, alert, inactive babies showed most facial activity. Tests situational/contextual variable of arousal state as a factor in gate theory. Score done in 3 second segments Originally for research. Being validated for clinical use.
COMFORT (Ambuel <i>et al.</i> , 1992)		Acute and prolonged	Term and older	Facial expression	Muscle tone Calm/agitated	Resp response MAP HR Alertness	Originally to assess pharmacological and psychological interventions. Developed for Paediatric Intensive Care Unit use, not specifically neonates. Used mainly in research

Score (ref)	Full name	Acute/ Chronic	Term/ preterm	Face	Body	Physiol	Notes
PIPP (Stevens <i>et al.</i> , 1996)	Premature Infant Pain Profile	Acute	Preterm >28 weeks	Facial expression	no	O2 HR	Research initially, now validated for use in clinical setting. GA and behavioural state accounted for.  Developed for use in NICU
IBCS (Craig <i>et al.</i> , 1984)	Infant body Coding System	Acute	Term and Preterm	No	Hand, foot, arm, leg, head, torso, motor activity.	No	Validated on response to heel lance. Facial activity increased with GA. Body movements less in preterm.
NIPS (Lawrence <i>et al.</i> , 1993)	Neonatal Infant Pain Score	Acute	Term and Preterm	Facial expression Cry Arousal	Limb positions	Resp pattern	Research tool tested on small number
PPS (Barrier <i>et al.</i> , 1989) (IPS)	Post op pain score (Infant Pain Scale)	Post op	Preterm and Term	Facial expression Cry quality Sucking Consolability Sociability	Spont motor activity Spont excitability Toe and finger flexion Global tone	Sleep in preceeding hour	Compared fentanyl to placebo Post op, minor procedures, 1-7 months, and narcotic effect
SUN (Blauer and Gerstmann, 1998)	Scale for Use in Newborns	Acute	Preterm and Term	Facial expression Behavioural state	Tone Movement	HR BP Breathing	Compared in publication to COMFORT and NIPS scales.



Score (ref)	Full name	Acute/ Chronic	Term/ preterm	Face	Body	Physiol	Notes
CRIES (Krechel and Bildner, 1995)	Crying Requires O2 Increased vital signs Expression Sleeplessness	Post op	Term	Facial Expression Crying Sleeplessness	No	O2 sats HR BP	Scored in same way as APGAR. Intended for clinical use.
LIDS (Horgan and Choonara, 1996)	Liverpool Infant Distress Score	Post op	Term	Facial expression Quality of cry Sleep	Spont movements Spont excitability Flexion of fingers and toes	No	Intended for clinical use.
DSVN (Sparshott M, 1996)	Distress scale for Ventilated Newborns	Acute	Preterm and Term	Facial Expression Colour	Body movements	O2 sats HR BP Temp	For assessment of procedural pain in ventilated newborns.
BPS (Pokela, 1994a)	Behavioral Pain Score	Acute	Preterm and Term	Facial expression	Body movements Response to handling Consolability Rigidity of limbs	No	Less response to tracheal suction after opiates, as measured by score.

Score (ref)	Full name	Acute/ Chronic	Term/ preterm	Face	Body	Physiol	Notes
PAT (Hodgkinson <i>et al.</i> , 1994)	Pain Assessment Tool	Post op	Term	Facial expression Sleep pattern Cry Colour Nurses perceptions	Tone Posture	Resps HR O2 BP	1 <sup>st</sup> 24 hours after surgery
(Rushforth and Levene, 1994)	Behavioural response to pain in healthy neonates	Acute	Preterm and Term	Brow-bulge Eye squeeze Nasolabial furrow Open lips Cry latency and duration	No	No	Response to Heel lance Brow bulge and nasolabial fold changes most consistent, and more often than cry.

Table 2-1 Scores for assessing pain in the newborn infant

## **2.3 Neuroendocrinology and Stress**

### **2.3.1 Stress concepts**

Hans Selye considered 'stress' to be the non-specific response of the body to any demand, and developed his whole theory of the 'general adaptation syndrome' in the early decades of the 20<sup>th</sup> century on the basis of this definition. His original concept of stress was distinct from that of distress, and he saw stress as a positive experience, as long as the demands placed on the body could be accommodated and coped with. The popular recent concept of stress involves a failure of adaptation. More recent research suggests that reactions to stress are specific, with particular definable patterns and that once a stressor exceeds a certain threshold, responses may become extreme and generalised. To various degrees, depending on the nature of the stressor, physiological and psychological compensatory mechanisms, which ultimately interact, are required to deal with the 'threat to homeostasis' that embodies 'stress'.

At the beginning of the 20<sup>th</sup> century, W. B. Cannon first highlighted the role of the sympathetic nervous system in mediating many of the more obvious physiological changes characteristic of 'highly stressed' organisms. The classic 'fight or flight' response typified by sympatho-adrenal activation is instigated by emotions such as fear, rage, and 'mental challenge' (Baillieres, 1987; Stratakis and Chrousos, 1995).

Besides emotion, physical stress in the form of actual or threatened tissue damage is a potent activator of the HPA axis and sympatho-adrenal system, as discussed in more detail below.

It is thought that the endogenous opioid system counteracts, buffers and modulates the HPA and sympathoadrenal systems.

Central to Seyle's theory of stress was the importance of the hypothalamo-pituitary-adrenocortical system (see fig 2.2). More recent work has identified the peptide, corticotrophin releasing hormone or factor (CRH), key to activation of the HPA axis and produced by the hypothalamus, as a central modulating neurotransmitter (see figs 2.2 and 2.3). In adult psychobiological studies, activation of the adrenocortical system is associated with negative emotions such as uncertainty, depression, withdrawal, and of being unable to cope. Chronic activation of the HPA axis has been shown in anorexia nervosa, panic anxiety, obsessive-compulsive disorder, chronic active alcoholism, excessive exercising, malnutrition, and sexually abused girls. The function of this adrenal response to emotional stimuli is thought to be feedback to the hippocampus, which is rich in corticosteroid receptors, for modulation of the central response to stress (Baillieres, 1987).

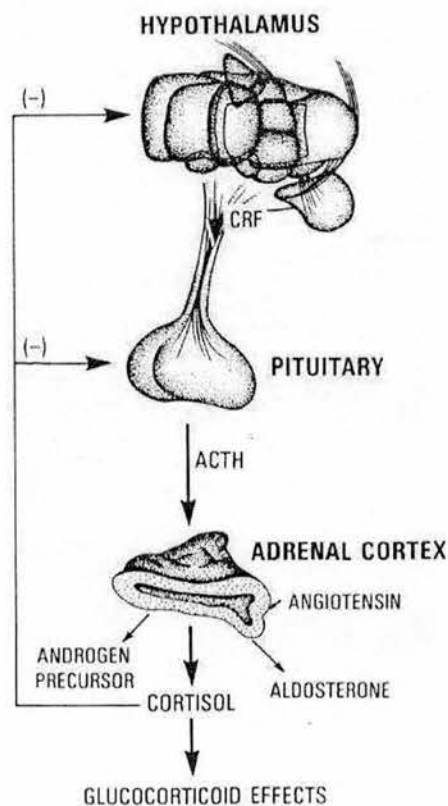


Figure 2-2 The Hypothalamo-Pituitary-Adrenal System

### 2.3.2 Neuroendocrine axis

The 'neuroendocrine axis' comprises the CNS (particularly the limbic system) and its activation and modulation of the HPA, sympathetic nervous, endogenous opioid, and related systems. Its role is to act 'in concert' with other physiological systems to maintain the integrity of the individual in the face of 'stress' or challenge.

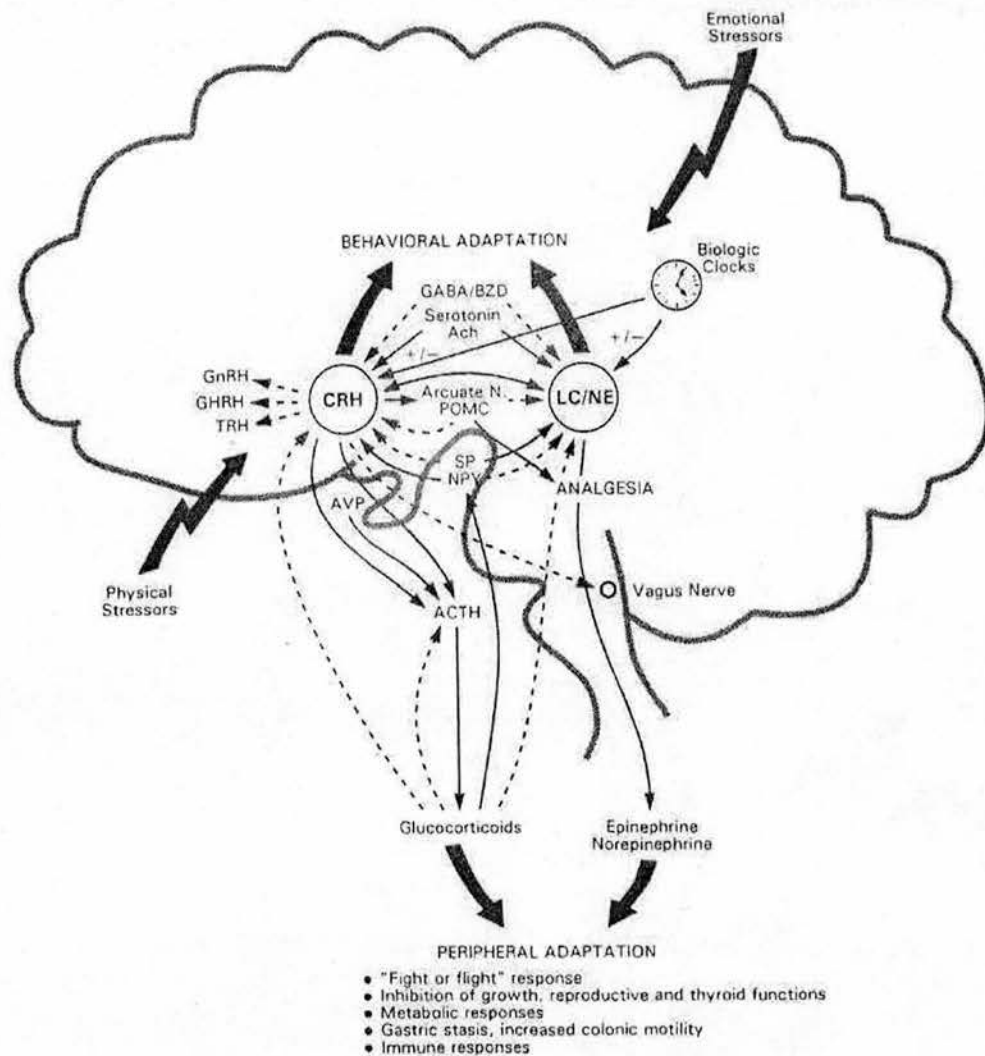


Figure 2-3: Representation of the 'stress system and neuro-endocrine axis' (Stratakis and Chrousos, 1995)

The neuroendocrine responses to stress may be considered as adaptive responses on the rising side of a bell-shaped curve. The fragmenting responses on the down side of the curve may represent the breakdown of adaptation in the face of excessive demands, with negative feedback loops disintegrating and damage supervening.

Table 2-2 : Stimuli that have been shown to activate the HPA axis

	Newborn	Children	Adults	Animals
<b>Sustained negative emotion</b>			•see above, and summerised in (Stratakis and Chrousos, 1995)	
<b>Acute painful procedures</b>	•see below (section 2.3.5)	• (Lewis and Thomas, 1990)		
<b>Restraint</b>	• (Malone <i>et al.</i> , 1985)			• (Collins <i>et al.</i> , 1984)
<b>IL-1, IL-2, TNF<math>\alpha</math>/ sepsis</b>		• (Nickels and Moore, 1989; Herschenfeld and Geffner, 1996)	• (Frayn, 1986)	• (Kapcala <i>et al.</i> , 1995)
<b>Physical injury (accident)</b>		• (Rainer <i>et al.</i> , 1999)	• (Barton, 1987; Frayn, 1986)	
<b>Surgical stress</b>	• see below (section 2.3.7)	• (Bouwmeester <i>et al.</i> , 2001; Bozkurt <i>et al.</i> , 2000)	• (Friedrich <i>et al.</i> , 1999)	
<b>Medical illness</b>	Unclear. Discussed in chapter 8	• (Deshpande <i>et al.</i> , 1993; Nickels and Moore, 1989)	• (Jurney <i>et al.</i> , 1987; Reincke <i>et al.</i> , 1995)	

Insulin tolerance tests are used widely in both adults and children to assess 'normal' cortisol response to hypoglycaemia. The fetus has been shown to mount a significant cortisol response to the acute stress of intrauterine transhepatic 'needling' (Giannakoulopoulos *et al.*, 1999).

### 2.3.3 Effects of HPA activation

Metabolic: enhanced hepatic gluconeogenesis, decreased insulin secretion and peripheral insulin resistance

Electrolyte and fluid balance, particularly sodium and fluid retention

Haemodynamic: increased vascular response to catecholamines

The HPAA also has complex interactions with the Hypothalamo-pituitary-gonadal, thyroid and growth axes.

### 2.3.4 HPA response to inflammatory mediators

The inflammatory mediators,  $\text{TNF}\alpha$ , IL-1 and IL-6 cause activation of the HPA axis alone and in synergy with each other. IL-6 causes major elevations of ACTH and cortisol, well above levels achieved with maximally stimulatory doses of CRH. It appears to act through stimulation of hypothalamic CRH and AVP secretion, and by direct effects at the pituitary and adrenocortical levels. Glucocorticoids are thus thought to play a major role in the stress-induced suppression of immune-inflammatory reaction (Stratakis and Chrousos, 1995). Extremely high levels of cortisol have been demonstrated in children with serious bacterial infections (mean peak cortisol concentration of  $1060 \text{ nmol L}^{-1}$  in bacterial meningitis) (Nickels and Moore, 1989).

In a study of adults with septic shock, plasma cortisol concentrations were increased markedly up to  $11,000$  (median  $1400$ )  $\text{nmol L}^{-1}$ . Patients with Gram-positive infections had increased cortisol levels compared with those who had Gram-negative infections (Schein *et al.*, 1990)

In a study of 13 neonates (GA 28-40 weeks) with septic shock, endotoxin was recovered from eight infants. Serum cortisol concentration from infants with endotoxemia ( $2500 \pm 1644 \text{ nmol L}^{-1}$ ) was significantly higher than that from infants without endotoxemia ( $1097 \pm 659 \text{ nmol L}^{-1}$ ). The highest single value noted was  $6350 \text{ nmol L}^{-1}$  (Togari *et al.*, 1986).

Herschenfeld & Geffner report a case of an 8 month old infant presenting with staphylococcus aureus sepsis who had a single sample of plasma with a cortisol value of 13,500 nmol L<sup>-1</sup>, that failed to respond to ACTH and subsequently fell to normal after 5 days of antibiotics (Herschenfeld and Geffner, 1996).

### 2.3.5 HPA response to acute procedural pain in the newborn infant

In the newborn infant, significant increases in cortisol (plasma or saliva) have been demonstrated following circumcision (Gunnar *et al.*, 1981; Talbert *et al.*, 1976; Williamson and Evans, 1986), venepuncture (Fiselier *et al.*, 1983; Mantagos *et al.*, 1991) and heel lance (Kurihara *et al.*, 1996).

### 2.3.6 Neuroendocrine response to medical illness

Adult ITU patients have higher cortisol and ACTH than controls, and there is a correlation between cortisol levels, severity of illness and mortality. There is also a lack of cortisol circadian rhythm in the patients with raised levels. The cortisol response to ACTH in the early stages of critical illness is frequently exaggerated, but when levels are extremely high, the response may be blunted or absent. There is also a shift away from the synthesis of androgens and mineralocorticoids towards glucocorticoids. There are significant parallels between the HPA changes in major depression and critical illness. It has been postulated that peripheral glucocorticoid receptor resistance may underlie exaggerated cortisol levels. There is down regulation of the pituitary-gonadal and pituitary-thyroid axes (Reincke *et al.*, 1995).

Endocrine stress responses have been recorded in infants beyond the newborn period with medical conditions such as bronchiolitis and gastroenteritis that are of a similar magnitude to post operative stress (Deshpande *et al.*, 1993). The literature on the cortisol response to medical illness in the newborn is described in appendix 1. Overall, there seem to be equal numbers of studies both demonstrating, and failing to demonstrate, a significant rise in cortisol in response to medical illness.



### 2.3.7 Neuroendocrine response to surgical stress

A great deal of neonatal pain research was inspired by a series of studies by Anand and co-workers in the late 1980s and early 1990s. He and colleagues described the metabolic and endocrine response in preterm and term newborn infants undergoing surgery. The results showed that newborn infants could mount a substantial endocrine and metabolic stress response, the main features of which were hyperglycemia and hyperlactataemia associated with the release of catecholamines and the inhibition of insulin secretion. They described specific differences between preterm and term neonates (Anand *et al.*, 1985b; Anand *et al.*, 1985a). The series of studies showed that the degree of anaesthesia/analgesia administered to newborn infants undergoing surgery had a direct effect on stress hormone levels, markers of metabolic stress and clinical outcome.

In a randomised controlled trial, preterm babies undergoing ligation of a patent ductus arteriosus were given nitrous oxide and d-tubocurarine, with ( $n = 8$ ) or without ( $n = 8$ ) fentanyl (an opioid analgesic drug) as part of the anaesthetic regimen. Major hormonal responses to surgery (changes in levels of plasma adrenaline, noradrenaline, glucagon, aldosterone, corticosterone, 11-deoxycorticosterone, 11-deoxycortisol, insulin/glucagon ratio, blood glucose, lactate, and pyruvate) were significantly greater in the non-fentanyl than in the fentanyl group. The urinary 3-methylhistidine/creatinine ratios were also significantly greater in the non-fentanyl group on the second and third postoperative days. Compared with the fentanyl group, the non-fentanyl group had circulatory and metabolic complications postoperatively. The findings indicate that preterm babies mount a substantial stress response to surgery under anaesthesia with nitrous oxide and curare and that prevention of this response by fentanyl administration is associated with an improved postoperative outcome (Anand *et al.*, 1987).

In a subsequent study, 36 neonates undergoing operations were randomised to two groups: one group received anaesthesia with nitrous oxide and curare alone, and the other was given

halothane in addition. Concentrations of metabolites and hormones were measured before and at the end of operation and at 6, 12, and 24 hours after operation, and the values compared between the two groups. Neonates given halothane anaesthesia showed decreased hormonal responses to operation, with significant differences between the two groups in the changes in adrenaline, noradrenaline, and cortisol concentrations and the ratio of insulin to glucagon. Changes in blood concentrations of glucose, total ketone bodies and non-esterified fatty acids were also decreased in neonates receiving halothane anaesthesia. Neonates given anaesthesia with unsupplemented nitrous oxide showed significantly greater increases in the urinary ratio of 3-methylhistidine to creatinine concentration and their clinical condition was also less stable during and after operation (Anand *et al.*, 1988).

Neonates who received deep anesthesia with sufentanil (an opioid analgesic drug) had significantly reduced responses of beta-endorphin, norepinephrine, epinephrine, glucagon, aldosterone, cortisol, and other steroid hormones; their insulin responses and ratios of insulin to glucagon were greater during the operation. The neonates who received lighter anesthesia (with halothane plus morphine) had more severe hyperglycemia and lactic acidemia during surgery and higher lactate and acetoacetate concentrations postoperatively. The group that received deep anesthesia had a decreased incidence of sepsis, metabolic acidosis, and disseminated intravascular coagulation and fewer postoperative deaths (none of 30 given sufentanil vs. 4 of 15 given halothane plus morphine (Anand and Hickey, 1992).

Graded hormonal and metabolic responses to differing degrees of surgical stress were then described in neonates in minor ( $n = 71$ ), moderate ( $n = 12$ ), and severe ( $n = 11$ ) stress groups. Clinical outcome following operation was also significantly different between the three stress groups (Anand and Aynsley-Green, 1988).

Further study revealed that neonatal stress responses to cardiac surgery are distinct from, and more extreme than, those seen in adult cardiac surgical patients. In one study, neonates who

died postoperatively tended to have higher stress responses than survivors (Anand *et al.*, 1990).

### 2.3.8 Effects of excessive neuroendocrine stress response

In the medical environment, humans may be subjected to procedures and interventions that evoke extreme stress responses. As described in the series of studies above, the effects span every system, and interventions that decrease stress responses and their parallel hormone levels have been associated with improved clinical outcome.

In animal studies, high levels of glucocorticoids have been found to exacerbate hypoxic-ischaemic neuronal damage with high levels associated with hippocampal damage (Sapolsky *et al.*, 1990; Sapolsky and Pulsinelli, 1985). Such observation has led to the proposal that in adults, pharmacological interventions that diminish the adrenocortical stress response may improve neurological outcome from stroke or cardiac arrest.

There is evidence that a severe and prolonged catabolic reaction to injury may be associated with an increased morbidity and mortality in high-risk adult patients. This subject, along with associated stress hormone responses, is reviewed by Anand (Anand, 1986).

### 2.3.9 Summary

The neuroendocrine system plays a vital role in preserving the wellbeing of the individual organism should it be subjected to physical, physiological or psychological 'stress'. The hypothalamo-pituitary-adrenal axis plays a pivotal role in this system. There is evidence of the newborn infant's ability to mount an appropriate neuroendocrine response to inflammatory processes, medical and surgical stress, and pain.

## **2.4 Measurement of salivary cortisol**

### **2.4.1 Physiological basis**

In the late 1950s and 1960s, Katz & Shannon were the first to publish results of the measurement of steroids in saliva (Katz and Shannon, 1969b; Katz and Shannon, 1969a). This work was taken further and applied in the clinical setting by the 'Tenovus' group based in Cardiff (Walker *et al.*, 1978; Walker *et al.*, 1990; Francis *et al.*, 1987; Riad-Fahmy *et al.*, 1980; Riad-Fahmy *et al.*, 1982; Riad-Fahmy *et al.*, 1983; Walker *et al.*, 1979) and Vining & McGinley (Vining *et al.*, 1983b; Vining *et al.*, 1983a; Vining and McGinley, 1986; Vining and McGinley, 1987) in the late 1970s and 1980s.

Salivary cortisol measurement by radioimmunoassay is now a widely used tool in both the clinical setting and in psychobiological studies of stress in infants, children and adults.

The easy stress-free, non-invasive nature of saliva collection makes it one of the most accessible of body fluids and it is of value in studying normal human physiology as well as pathology (Vining and McGinley, 1986; Vining and McGinley, 1987). Salivary cortisol concentration has been found to be directly proportional to the serum unbound cortisol concentration (Vining *et al.*, 1983a) both in normal men and women and in women with elevated cortisol-binding globulin (CBG). The correlation with plasma cortisol is excellent in dynamic tests of adrenal function (dexamethasone suppression and ACTH stimulation), in normal subjects and patients with adrenal insufficiency, in tests of circadian variation and randomly collected samples. The relationship between salivary and serum total cortisol concentration in adults is non-linear with a more rapid increase in salivary concentration once the serum CBG is saturated. The rate of equilibrium of cortisol between blood and saliva is less than 5 minutes. Further work has suggested that unconjugated steroids enter saliva by diffusing through the cells of the salivary glands and that their concentration in

saliva does not depend on the rate of saliva production (Vining *et al.*, 1983a). The above data, combined with the accessibility of saliva, lead researchers to suggest that salivary cortisol may be a more appropriate measure for the clinical assessment of adrenocortical function than serum cortisol (Vining *et al.*, 1983b).

Walker *et al* described a direct radioimmunoassay (RIA) for cortisol in 10-microliter volumes of parotid or whole saliva. Binding proteins were presumed to be absent from these fluids, as demonstrated by the excellent correlation between results for samples assayed directly and by a comparison procedure involving extraction with 1,2-dichloroethane. Comparison of cortisol concentrations in plasma, parotid saliva, and whole saliva in subjects undergoing investigations for assessing adrenal function, including stimulation with ACTH and suppression with dexamethasone, indicated that changes in plasma cortisol concentration were accurately and almost immediately reflected in saliva from either the parotid gland or whole saliva. A marked circadian rhythm was also demonstrated for cortisol in parotid-gland saliva and whole saliva (Riad-Fahmy *et al.*, 1983; Walker *et al.*, 1978). The same team demonstrated the successful clinical use of salivary hormone measurement in the monitoring of patients with Congenital Adrenal Hyperplasia (Walker *et al.*, 1979).

Riad-Fahmy *et al* also found, as above, that cortisol concentration in saliva was independent of salivary flow rate. As with Vining & McGinley, they described a disproportionate increase in salivary cortisol when plasma levels exceed 500nmol/l as this is the level at which CBG binding sites are saturated in adults, resulting in rapidly increasing free cortisol in the circulation (Riad-Fahmy *et al.*, 1982).

The measurement of free cortisol in plasma requires sophisticated techniques such as equilibrium dialysis or ultrafiltration. These techniques are not suitable for routine use. As salivary cortisol mirrors levels of free plasma cortisol, but is technically more accessible, it offers an attractive means of assessing the biologically relevant product of the HPA axis. RIA of salivary cortisol also circumvents the physiological, pathological, and

pharmacological changes due to corticosteroid-binding globulin alterations (Laudat *et al.*, 1988).

Adult salivary cortisol levels range from 1.3 to 27.6% of total plasma levels (Tunn *et al.*, 1992; Vining *et al.*, 1983b). Vining and McGinlay found that salivary cortisol was about 30% less than unbound serum cortisol and presumed the deficit to be due to the partial conversion of cortisol to cortisone by 11- $\beta$ -hydroxysteroid dehydrogenase type 2 in the salivary gland (Vining *et al.*, 1983b). This is discussed further below.

The findings described above have subsequently been reproduced by various authors who advocate the use of salivary cortisol measurement in the clinical setting in infants, adults and children (Bober *et al.*, 1988; Castro *et al.*, 2000; Hiramatsu, 1981; Lo *et al.*, 1992; Peters *et al.*, 1982; Schmidt, 1998; Tunn *et al.*, 1992; Umeda *et al.*, 1981).

Shimada *et al.* described the adaptation of a commercially available ELISA kit for the measurement of salivary cortisol, illustrating the accessibility of the tool to even non-specialist laboratories. In 35 young children, circadian rhythms of salivary cortisol levels were determined by the commercially available kit with a minor modification (Al Ansari *et al.*, 1982; Shimada *et al.*, 1995).

#### 2.4.2 Cortisol and cortisone

In humans, cortisone derives mainly from the oxidation of the 11-hydroxyl function of cortisol into 11-ketone by 11- $\beta$ -hydroxysteroid dehydrogenase type 2. In the adult, this enzyme is located in mineralocorticoid target tissues, principally kidney, colon, and parotid gland, and most of the conversion takes place in the kidney. The conversion of cortisone to cortisol takes place mainly in the liver by the action of 11- $\beta$ -hydroxysteroid dehydrogenase type 1. In the adult, cortisol exceeds cortisone 10 fold. 11- $\beta$ -hydroxysteroid dehydrogenase type 2 is present in many tissues in the fetus and in the placenta (Stewart *et al.*, 1994; Stewart *et al.*, 1995). Thus in the fetus, cortisone is present in concentrations 3 times that of cortisol

(Nahoul *et al.*, 1988). The factors that regulate the activity of the enzyme complexes and so the balance between cortisol and cortisone are not fully understood. It is known that in infants born even as prematurely as 24 weeks, following birth, their enzyme activity switches to a more adult type pattern, with cortisol predominating in the plasma (Kojima *et al.*, 1981; Sippell *et al.*, 1978a). Cortisol:cortisone ratios take some time to become completely similar to more mature infants and adults, implying relatively high 11- $\beta$ -hydroxysteroid dehydrogenase type 2 activity (Fujitaka *et al.*, 1997).

Brooks and Brooks examined the possibility of conversion of cortisol to cortisone in the salivary gland in adults by measuring serum free and salivary cortisol and cortisone. In response to ACTH, cortisone in saliva increased to a level twice that of total plasma cortisone which showed a minimal increase. Basally, salivary cortisone was higher than free plasma cortisone. Salivary cortisone was more dependent on plasma cortisol than plasma cortisone. Salivary flow was also seen to increase the cortisol:cortisone ratio ie salivary cortisone is decreased by increased saliva flow (Brooks FS, 1984). These results suggest significant 11- $\beta$ -hydroxysteroid dehydrogenase type 2 activity in the adult salivary gland. This inference is backed by the work of Meulenberg and Hofman (Meulenberg and Hofman, 1990). There appears to be no literature describing the activity of 11- $\beta$ -hydroxysteroid dehydrogenase type 2 in the preterm infant's salivary gland.



### 2.4.3 Review: Salivary Cortisol data in infants.

Various studies have used salivary cortisol for the assessment of adrenocortical function in infants of several months and older. Fewer studies have focused on the newborn population. The following is a review of the literature containing salivary cortisol data from the first month of life. All units have been converted to  $\text{nmol L}^{-1}$  for ease of comparison. This conversion is based on  $1\mu\text{g dL}^{-1}$  of cortisol being equivalent to  $0.03625\text{ nmol L}^{-1}$ . Summary data are presented in table 2.3.

The literature falls into three main categories:

1. Establishment of normal data including emergence of circadian cortisol rhythm.
2. Psychobiological studies of new-born infants' stress responses.
3. Studies relating to other clinical areas.

Publications including results from pre-term infants within these categories are:

(Antonini *et al.*, 2000)

(Azcarate *et al.*, 1997)

(Bettendorf *et al.*, 1998)

(Calixto *et al.*, 2002)

(Vaughn *et al.*, 1996)

#### 2.4.3.1 Normal data and circadian rhythm

In 1983, Price *et al* were among the first to publish salivary cortisol data from young infants (Price *et al.*, 1983). Eight infants were followed at monthly intervals for six months, the primary aim being to establish the age of onset of circadian rhythm in cortisol secretion. This was found to be on average the third month of life, and to follow the emergence of a night-day sleep pattern. It was noted that levels were higher and more variable in the first month of



life. There were however, no details of results from the very early neonatal period. Saliva was collected by mucous extractor after stimulation of saliva flow with citric acid and analysed by radioimmunoassay. Values ranged from 'not detectable' to 50 nmol L<sup>-1</sup>.

Francis et al studied salivary cortisol in eight term, two-day-old infants in 1987. Samples were taken 2 hourly over 24 hours. Two maxima were identified in each day. No correlation was found between salivary cortisol level and sleep or wake state, arousal or environment. There was a good correlation between saliva and plasma levels of cortisol in a separate group of 36 normal term babies ( $r = 0.83$ ). Daily variations were numerous and marked (21-42%). It was suggested that cortisol levels were more variable than in adults because saturation of CBG binding sites impaired the ability of CBG to buffer oscillations of cortisol in the circulation. Collection was by De Lee suction catheter over five to ten minutes with citric acid. Saliva values ranged from 2.5 to 57.4 nmol L<sup>-1</sup> Plasma values ranged from 29-507 nmol L<sup>-1</sup> (Francis *et al.*, 1987).

In 1991 Spangler et al set out to establish basic information on salivary cortisol levels and examine the emergence of cortisol circadian rhythm. He studied eleven newborn infants and fourteen 3-7 month olds. Infants were assessed at 2-7 days of age then at 3, 5 and 7 months. Four samples were taken within each 24-hour period. It was found that adult type circadian rhythm was established by 3 months and was paralleled by a 24-hour sleep-wake cycle. Feeds were associated with lower cortisol levels in infants over three months of age but not new-borns. In infants greater than 3 months of age, the more sleep in preceding hours, the higher the cortisol level, but again no such association was seen in newborns. An interesting correlation between infant and maternal salivary cortisol was found, including 10 bottle-fed babies. There was high intra-subject stability in variations in cortisol levels, and so the authors recommend taking into account individual baseline as well as sleeping and feeding behaviour when interpreting salivary cortisol results. A range of 0.28-27.6 nmol L<sup>-1</sup> was found for newborns (Spangler, 1991).

In 1995 Kiess et al sought to describe salivary cortisol levels throughout childhood. Ten infants less than one year old were studied. Samples were taken at 0800, 1300 and 1800 in one day only. It was concluded that there was no evidence of circadian rhythm before nine months of age. In some samples from young babies, evening samples were found to be as many as five times higher than the morning samples. Overall, the highest values were found in those aged <one year. Saliva was collected using a ball and pipette with no citric acid and analysed by immunofluorescent assay. The range was found to be <2 to > 100 nmol L<sup>-1</sup> (Kiess *et al.*, 1995).

Santiago et al sought to establish the time of emergence of circadian rhythm in cortisol secretion in nine term infants in 1996. Samples were taken in the morning, afternoon and evening at 2, 4, 8, 12, 16, 20 and 24 weeks. On average, circadian rhythm took 8 weeks to emerge. Samples were collected by suction from the floor of the mouth using a tube and syringe after stimulation with citric acid, and analysed by RIA. The range of values was 'not detectable' to 28 nmol L<sup>-1</sup> (Santiago *et al.*, 1996).

In 1997, Antonini et al investigated the time of emergence of circadian rhythm in cortisol secretion in preterm infants and its relationship to sleep pattern. Nine preterm infants, gestational age 31-34 weeks, were followed from birth to 24 weeks. Samples were collected twice in the first month, then monthly in the morning and evening, and analysed by RIA. Circadian rhythm for cortisol secretion emerged at a median of 8 and a mean of 9.3 weeks. No numerical results were given in this study (Antonini *et al.*, 2000).

Klug et al established parallel normative data for salivary cortisol and 17-hydroxyprogesterone (17-OHP) levels in healthy neonates. Cortisol and 17-OHP levels in saliva samples from 119 healthy neonates (55 girls, 64 boys) were measured using in-house time-resolved fluorescent immunoassays. Saliva samples were obtained using a saliva collecting tube three times a day on the first or second day of life. Gender and gestational age did not influence salivary cortisol and 17-OHP levels. No significant circadian rhythm of

salivary hormone levels was detected in this group of newborns. However, body mass index, arterial cord blood pH and time of saliva sampling significantly influenced salivary hormone levels (Klug *et al.*, 2000).

Calixto *et al* sought to determine correlations between plasma and salivary cortisol levels in preterm infants in the basal state and after ACTH stimulation during the first week of life. Infants ( $n = 48$ ) were given ACTH and saline solution; each injection was separated by 24 hours. Salivary and plasma cortisol levels correlated at baseline ( $r = 0.67$ ) and 1 hour after ACTH stimulation ( $r = 0.40$ ). ACTH increased cortisol levels in plasma from a mean of  $340 \text{ nmol L}^{-1}$  to  $827 \text{ nmol L}^{-1}$  and in saliva from  $27.6 \text{ nmol L}^{-1}$  to  $71 \text{ nmol L}^{-1}$  (Calixto *et al.*, 2002).

#### 2.4.3.2 Psychobiological studies of stress response

In 1993, Spangler examined physiological correlates of behavioural organisation in the newborn. He measured salivary cortisol and heart rate parameters in response to the Neonatal Behavioral Assessment Scale (Brazelton, 1984). 42 babies were assessed between the first and seventh days of life. A significant cortisol increase was demonstrated in response to mild aversive stimulation in a subgroup of babies. Infants with low orientation scores showed larger increases in cortisol than the highly orientated babies who showed little or no increase in cortisol in response to the assessment. Newborns with high orientation (or behavioural organisation) had high basal cortisol levels and vice versa. The range was  $15.2\text{-}35.8 \text{ nmol L}^{-1}$  (Spangler and Scheubeck, 1993).

Davis and Emory investigated sex differences in neonatal stress reactivity by examining salivary cortisol, heart rate parameters, and behaviour assessment. Eighteen males and eighteen females were examined at about forty hours of age. In response to the mild stress of the assessment, males had a greater cortisol increase, and females greater heart rate and behaviour changes. Saliva was collected by DeLee suction catheter with citric acid solution.

Values for salivary cortisol were (mean  $\pm$  SD) 29.5  $\pm$  32.8 nmol L<sup>-1</sup> for females and 30.1  $\pm$  36.4 nmol L<sup>-1</sup> for males at baseline (Davis and Emory, 1995a).

In 1992, Magnano et al studied the adrenocortical response to heel-stick and physical examination of infants born to mothers who abused cocaine during pregnancy compared to controls (Magnano *et al.*, 1992). The cocaine-exposed infants were reported to have similar basal salivary cortisol levels to non-cocaine exposed controls, but a suppressed response to both heel stick and examination. Saliva was collected by DeLee suction catheter without citric acid and cortisol measured by commercial RIA kit. Mean salivary cortisol levels in controls were 15.4 nmol L<sup>-1</sup> basally, 23.7 nmol L<sup>-1</sup> following examination and 35.9 nmol L<sup>-1</sup> following heel stick.

In 1995, Gunnar et al described a relationship between response to heel-stick and emotional temperament at 6 months. Salivary cortisol (before and 19 minutes after heel-stick) was measured along with heart rate parameters including vagal tone (calculated from heart rate changes), and behavioural state. 50 normal term infants were assessed at varying postnatal ages, averaging about 45 hours. Saliva was collected onto a cotton swab using citric acid and analysed by commercial RIA kit. Although baseline vagal tone appeared to predict cortisol response to heel-stick, no overall relationship was seen between vagal tone and salivary cortisol levels. Values were mean (range) for baseline: 11.0 (1.7-24.8) nmol L<sup>-1</sup> and for post heel-stick: 34.2 (4.7-121.4) nmol L<sup>-1</sup> (Gunnar *et al.*, 1995).

In 1995, Gunnar reviewed the work of her own group and others in 'The human infant's adrenocortical response to potentially stressful events.' In this review, she suggested an approach to differentiating changes in cortisol levels due to 'stress' as opposed to normal 'non-stressed' fluctuations. She suggested that the ratio of unbound to bound plasma cortisol is the key concept. Based on adult data, at a total plasma cortisol concentration of 440 nmol L<sup>-1</sup>, the ratio of unbound to bound cortisol suddenly increases, as all the plasma cortisol binding sites are saturated. (At this point, any increase in cortisol is an increase in the

biologically active and available free hormone.) The free plasma cortisol in her work was taken to be equivalent to salivary cortisol. Therefore, salivary cortisol values were used in these calculations. Applying this to a meta-analysis of studies of cortisol response to stimuli ranging from undressing and weighing to heel-stick and circumcision, only circumcision and high-risk delivery clearly and consistently evoked a response of this magnitude. This model is helpful when considering the wide range of adreno-cortical responses, wide intra-subject fluctuation and seemingly conflicting data regarding auxologous and behavioural correlations. Disregarding levels of total equivalent plasma cortisol below  $440 \text{ nmol L}^{-1}$  as not indicating significant stress, Gunnar re-examined data from a number of studies and found that differences in behaviour appeared or were most salient when cortisol exceeded this level. She also concluded that adrenocortical activity is not merely a reflection of behavioural distress, and that both withdrawing into quiescent states and crying serve as coping behaviour (Gunnar, 1989).

In 1996, Kurihara et al examined the adrenocortical and behavioural response to heel-stick, this time with the aim of describing the stabilising effect of a recording of maternal heart beat on those responses. As well as salivary cortisol, salivary DHEA and DHEAS were measured. 131 normal term infants were examined at 5 days of age, 1hr after feeding. Samples were taken 5-10 mins before, and 20 mins after, heel-stick. Ratio of 'before' to 'after' heel stick salivary cortisol was used as an index of stress. Samples were analysed by commercial RIA kit. Saliva was collected using a dental role in all, and citric acid in some cases. Maternal heartbeat was found to reduce the grade of variation of cortisol response to stress, and Japanese drum at the same frequency did not. There was also some decrease in behavioural response. A sub-group of five infants was studied over 24 hours and no evidence of circadian rhythm was found. The range of values was 'not detectable' to  $69.0 \text{ nmol L}^{-1}$  (from graph) (Kurihara *et al.*, 1996).

#### 2.4.3.3 Other clinical areas

In 1996 Vaughn et al used salivary cortisol as a measure of effectiveness of opiate therapy in post-operative infants. Continuous infusion was compared with intermittent boluses of fentanyl. Saliva was collected for cortisol measurement and a pain score carried out at 2, 8 and 24 hours after surgery. Thirty-six infants between 32 and 52 weeks gestation were studied. There was a good correlation between pain score and salivary cortisol level. Salivary cortisol and pain scores were found to be similar in the two study groups, but the infusion was found to be clinically safer. Saliva was aspirated from the floor of the mouth after lemon glycerine on a swab to stimulate flow and analysed by commercial RIA kit. The range of results, all post operatively, was 50-265 nmol L<sup>-1</sup> (Vaughn *et al.*, 1996).

Azcarate et al published an abstract in 1997 with salivary cortisol results from 18 babies on the third day of life. The study was designed to establish normal daily variations and the impact of gestational age and severity of disease. 3 hourly samples were collected over 24 hours using citric acid. Four healthy term, seven healthy pre-term and seven sick preterm infants were studied. There was found to be great variability of salivary cortisol levels, with no fixed pattern. No difference was found between term and preterm infants and no difference between sick and well infants. Values ranged from medians of 1.1 to 172.1 nmol L<sup>-1</sup> (Azcarate *et al.*, 1997).

In 1989, Magnano et al raised the important issue of milk feeds possibly confounding cortisol measurement in salivary cortisol studies in young infants. Cortisol was measured by RIA in three common milk formulas and breast milk. Levels ranged from 45.2 to 2284.1 nmol L<sup>-1</sup> on straight milk, and 0.6 to 4.1 nmol L<sup>-1</sup> after de-fatting and extraction. Thus, many interfering factors are present in milk as well as cortisol itself, and this should be taken into account in planning for and interpreting salivary cortisol measurement in infants (Magnano *et al.*, 1989).

Bettendorph et al set out to test the practicability of sequential cortisol determinations in saliva of low birth weight neonates and to evaluate the impact of systemic and inhaled glucocorticoid therapy on saliva concentrations of cortisol in preterm neonates with bronchopulmonary dysplasia (BPD). Salivary cortisol levels were measured by RIA in saliva samples from 10 full-term and 10 preterm healthy neonates and from 20 preterm neonates with BPD during systemic [dexamethasone (DEX); n = 10] or topical steroid therapy [budesonide (BUD); n = 10]. Saliva samples of each individual were collected on 3 consecutive days at 06.00, 12.00, 18.00 and 24.00 h. Salivary cortisol levels in preterm neonates treated with steroids were significantly lower than those measured in healthy preterm neonates (Bettendorf *et al.*, 1998).

#### 2.4.4 Summary

There exists a considerable amount of data about cortisol in the saliva of adults, and its relationship to plasma cortisol. There is some comparable data from term infants but little from preterm or sick infants.



Auth/ ref	Year	Aims	Pop	nos	age	Collect	Freq.	Sub-group	max	min	Median/ mean	Findings
Antonini (Antonini <i>et al.</i> , 2000)	2000	Circadian rhythm and sleep patterns in preterm infants	31-34 weeks GA	9	2,4,8,16,20,24 weeks				14 est	2 est		Sleep and cortisol rhythm emerge together at 8-12 weeks.
Azcarate (Azcarate <i>et al.</i> , 1997)	1997	Daily variation. Impact of GA. severity of disease	healthy t healthy p sick p	18	3 days		3hrly		46.9 114.5 172.1	1.1 4.7 9.1	19.7 25.0 37.4	High intra subject variability No diff t (term)+p (preterm), sick or well
Betendorph (Betendorf <i>et al.</i> , 1998)	1998	Normal values of cortisol and related steroids in term and preterm infants	Term Preterm (26-31) BPD with steroids	10 10 20	2 15-39 14-32 20-39 days	Tube +citric	3 consecutive days. 4X/day	Term  Preterm	60.6  52.1	0.8  0.6	6.5med  5.5med	Salivary cortisol significantly lower in steroid treated infants. Lower cortisol in healthy preterm than term. No correlation with sleep state or arousal. Considerable variation.
Calixto (Calixto <i>et al.</i> , 2002)	2002	Saliva related to plasma cortisol and after ACTH in preterm infants	26-33 wks GA stable	48 infants 144 samples	3-7 days	Tube+citric	Pre+post ACTH				27.6	R=0.68 pre ACTH between saliva and plasma. Significant change in saliva and plasma post ACTH but less tight correlation.
Davis and Emory (Davis and Emory, 1995a)	1995	Sex differences in stress reactions	Term normals	36	19-56 hours mean 40	Suction tube	5x at examination		Estimated upper : 110	basal	29.5 female 30.1 male	Discrimination between sexes possible by stress response to examination.



Auth/ ref	Year	Aims	Pop	nos	age	Collect	Freq.	Sub-group	max	min	Median/ mean	Findings
Francis (Francis <i>et al.</i> , 1987)	1987	Adrenocortical activity in term newborn infants Circ rhythm Relate to plasma	Term	8	2 days	2hrly	Asp +citric acid		57.4	2.5		Considerable variation. 21-42%. R=0.83. No relationship between arousal and cortisol.
Gruar (Grauer, 1989)	1989	Lighting, behavioural state + salivary cortisol.	Term Healthy	99	0.5-34 hours	Daily	Citric acid + suction trap				Mean est 27	Reduction in stress in some infants in intermittent compared to continuous light.
Gunnar (Gunnar <i>et al.</i> , 1995)	1995	Relationship between stress reaction to heelstick and later emotional temperament	Term normals	50	Average 45 hours Wide range	Cotton swabs	19 mins after heelstick	Pre Post heelstick	24.8 121.4	1.7 4.7	11 34.2 Means	Baseline vagal tone predicted response to heelstick.
Kiess (Kiess <i>et al.</i> , 1995)	1995	Describe salivary cortisol in childhood		10<1 yr	All ages	Ball+pipette	0800, 1300, 1800		>100	<2		No circ<9mo Evening samples sometimes 5 X morning levels
Klug (Klug <i>et al.</i> , 2000)	2000	Normal values for cortisol and 17OHP and diurnal rhythm	Term healthy	119	1 or 2 days	Cotton swab (salivette)	3 x per day	Day1 Day2	117	3.3	39.3 26.9	Cortisol related to cord pH in 1 <sup>st</sup> 24 hours. No sex diff or circ rhythm.

Auth/ ref	Year	Aims	Pop	nos	age	Collect	Freq.	Sub-group	max	min	Median/ mean	Findings
Kurihara (Kurihara <i>et al.</i> , 1996)	1996	Effects of maternal heart beat on cortisol+behavioural response to heelstick	Term	57 cont 41hb 31dru m	5days old	Dental role	20 mins after heelstick		104.8	nd		5 infants: no circadian rhythm Maternal heart beat reduced cortisol response Some reduction in behavioural response too. 0.7 cor coeff. n=72
Lewis and Thomas (Lewis and Thomas, 1990)	1990	Cortisol response to inoculation	Term well	20 25 24	2 4 6 months	Citric +cotton swab	Pre and 15 mins post		36 est stim	19 est basal		Examination and inoculation stimulated cortisol rise in parallel to behaviour. Basal cortisol neg correlation with increase in cortisol.
Magnano (Magnano <i>et al.</i> , 1992)	1992	Compare salivary cortisol in cocaine and non-cocaine exposed infants in NICU	30-37 weeks, well at test time	11 cocai ne expos ed. 35 norm al.	5-53 days	Pre and 30 mins post heelstick		Basal Non- invasive stressor Invasive stressor			15.4  23.7  35.9	No diff in basal cortisol between groups. Lower cortisol after exam and heelstick in cocaine exposed.
Price (Price <i>et al.</i> , 1983)	1983	Circ rhythm	Term	8	Birth to 6 mo	Mucous extractor	4 sample s a day		50	nd		Circadian rhythm by 3 <sup>rd</sup> month
Santiago (Santiago <i>et al.</i> , 1996)	1996	Circadian rhythm investigated	term	9	2,4,8,1 2,16,20 ,24wks	Tube+syr inge	TID		28	nd		Circ at 8 weeks Some at 2,4,8,12,20 weeks

Auth/ ref	Year	Aims	Pop	nos	age	Collect	Freq.	Sub-group	max	min	Median/ mean	Findings
Shimada (Shimada <i>et al.</i> , 1995)	1995	Validate ELISA Apply to circadian rhythm	2.5yrs 20 ex prems	35	One off	Spit or pippette	4hrly		62.1	0.28		ELISA works Ex-prems develop same levels and rhythm R=0.857
Spangler (Spangler and Scheubeck, 1993)	1993	Relate NBAS to salivary cortisol	Term infants	42	1-7days mornin g	Gauze swab			35.8	15.2	18.2pre 26.7 post exam	Orientation predicted by cortisol. Low correlation with irritability. High cortisol response in poorly oriented
Spangler (Spangler, 1991)	1991	Basic info on salivary cortisol Correlate sleep, feeds, maternal	newborn infants	11 14	2-7days 3,5,7 mo	Gauze swabs	4 hrly		27.6 19.3	0.28 2.8		Circadian by 3 months Feed decreases, sleep increases in older infants Baby and mat cortisol correlate
Taylor (Taylor <i>et al.</i> , 2000)	2000	Effect of delivery on stress response to inoculation	Normal term	46	8 weeks	Cotton roll	Pre and 20 mins post				1.2	Salivary cortisol and crying greatest in those with assisted delivery.
Vaughn (Vaughn PR, 1996a)	1996	Compares fentanyl inf w bolus	36-52 PCA weeks post op	36		suction	2,8,24 hrs post op		265	50		Correlation between pain score+cortisol

Table 2-3 Summary of literature on salivary cortisol levels in newborn infants

## **2.5 Adrenocortical function in the perinatal period.**

### **2.5.1 Ways of assessing adrenocortical function**

- 1) Total plasma cortisol measured by
  - a) Radioimmunoassay +/- extraction (Most studies in the past 20 years)
  - b) Immunofluorescent assay +/- extraction (Economou *et al.*, 1993)
  - c) Chromatography + UV detection (Fujitaka *et al.*, 1997)
  - d) Other, infrequently used methods eg MS or protein binding method of Murphy (Anders *et al.*, 1970)
- 2) Basal total cortisol values
  - a) random (Hanna *et al.*, 1997; Heckmann *et al.*, 2000)
  - b) morning (Hanna *et al.*, 1997) or timed (Economou *et al.*, 1993)
  - c) as part of cross sectional study (Heckmann *et al.*, 1999)
  - d) as part of longitudinal study (Doerr *et al.*, 1988)
- 3) Response to ACTH
  - a) High dose ('traditional') (Watterberg and Scott, 1995)
  - b) low dose ('physiological') (Korte *et al.*, 1996)
- 4) Response to CRH (Ng *et al.*, 1997b)
- 5) Metyrapone test (Alkalay *et al.*, 1990)
- 6) Cortisol production rates (Metzger *et al.*, 1993)
- 7) Saliva sampling (Bettendorf *et al.*, 1998)
- 8) Free plasma cortisol (Rokicki *et al.*, 1990)
- 9) With or without the measurement of

- a) Steroid precursors (Doerr *et al.*, 1988)
- b) Steroid metabolites (Fujitaka *et al.*, 1997)
- c) Cortisol binding globulin (Arnold *et al.*, 1998)

## 2.5.2 Key questions addressed by the literature

- 1) Are cortisol levels appropriate for clinical setting?
- 2) Are cortisol levels related to long-term clinical outcomes?
- 3) Are low cortisol levels related to specific disease processes such as hypotension or chronic lung disease?
- 4) Are high levels of cortisol harmful?
- 5) What processes underlie adrenocortical insufficiency?
  - a) Enzyme activity
  - b) Maturation of HPA regulation
  - c) Target tissue responsiveness
- 6) Can endogenous levels of cortisol be inappropriately high?
- 7) In light of all of the above, is exogenous steroid therapy likely to be
  - a) Required?
  - b) Effective?
  - c) How can the subgroup of patients most likely to benefit be identified?
- 8) How is adrenocortical function affected by
  - a) Antenatal steroid therapy?
  - b) Postnatal steroid therapy?

### 2.5.3 Possible reasons for disparity of findings in literature regarding cortisol levels

The literature on cortisol levels in the newborn infant over the past decades is extensive and is summarised in table 2-4. The results of the many studies often appear to be conflicting and difficult to compare. Here is an overview of some of the reasons:

1. The analysis of samples is often from **heterogeneous groups of infants**, ie a mixture of well and ill infants, of differing gestational ages, taken from infants on varying postnatal days. Only more recent publications attempt to define degree of illness severity, and more recently still attempt to employ validated illness severity tools. Prior to this, many studies talked simply of, for example, preterm compared to term neonates.
2. **Extraction** procedures with variable or low recovery, eg 65-85% in some publications. This is an issue particularly in earlier studies, when highly specific RIA was not available and so prior purification or extraction was necessary.
3. **Non-specific RIA.** RIA performed without prior purification could lead to overestimation of cortisol levels due to crossreactivity of the antibody with other steroids present in plasma. Even relatively recent publications quote high cross-reactivities;
  - a. 20% cross reactivity with cortisone (Kari *et al.*, 1996)
  - b. 18-30% cross reactivity with 11DOC, corticosterone, cortisone (Banks *et al.*, 2001a)
  - c. cross reactivity with cortisone, as 'unknown' (Huysman *et al.*, 2000)
  - d. no cross reactivities quoted (Hanna *et al.*, 1997)
  - e. 65% cross reactivity with 21-deoxycortisol (Thomas *et al.*, 1986)
  - f. 23 % cortisone and 28% 11-deoxycortisol cross reactivities (Hughes *et al.*, 1987)

The point has been made that many cortisol assays, including commercially available kits, are validated for adult serum and may give misleading results when applied to

neonatal serum (Murphy, 1980). Murphy suggests that many published cortisol values for newborn infants are too high owing to the use of methods insufficiently specific. Thus, authors who use methods such as HPLC to purify samples prior to RIA claim that their values are more representative than those using no extraction or purification procedures (Nomura, 1997).

4. The existence of a subgroup of infants with very poor capacity to produce cortisol, eg enzyme immaturity, may bring down the means/ medians of those infants with appropriate responses. Some authors have identified such 'outliers'. Korte found, along with others, a 'bimodal' distribution of cortisol results, with a subgroup of infants who, for whatever reason, seemed to have low cortisol levels. (Korte *et al.*, 1996)
5. **Use of opiates or sedation.** It is known that opiates can significantly reduce mean plasma cortisol levels (Barker and Rutter, 1996; Vaughn *et al.*, 1996). Many studies are carried out on ventilated infants, but no such studies record whether sedation was used.
6. **Small numbers of samples.** Recent highly specific yet low-volume RIAs have allowed 'micro' sampling when previously, large volumes of plasma were required for each measurement, making plasma sampling technically and ethically difficult.
7. **Small numbers of subjects.** With micro-sampling and salivary sampling, data are increasingly available from larger numbers of infants. Table 2-4 illustrates that many studies were carried out on relatively small groups of subjects. Larger numbers appear in cross sectional and more recent studies.
8. Studies frequently compare 'mean cortisol' values. The **distribution of cortisol values** is almost always highly skewed or bimodal, however, making this representation misleading, where medians or log transformation might be more representative (Heckmann *et al.*, 1999; Watterberg *et al.*, 2000).

## 2.5.4 Perinatal adrenal development

### 2.5.4.1 *The fetal adrenal gland*

The developmental and functional biology of the primate fetal adrenal cortex have been reviewed by Mesiano & Jaffe (Mesiano and Jaffe, 1997). The unique characteristics of the primate (particularly human) fetal adrenal were first realized in the early 1900s. The unusual architecture of the human fetal adrenal cortex, with its unique and disproportionately enlarged fetal zone, its compact definitive zone, and its dramatic remodeling soon after birth captured the interest of developmental anatomists. Many detailed anatomical studies describing the morphology of the developing human fetal adrenal were reported between 1920 and 1960, and these morphological descriptions have not changed significantly.

Elegant experiments during the 1950s and 1960s demonstrated the central role of the primate fetal adrenal cortex in establishing the oestrogenic milieu of pregnancy. Those findings were among the first indications of the function and physiological role of the human fetal adrenal cortex and led to the proposal of the concept of the 'feto-placental unit', in which DHEAS produced by the fetal adrenal cortex is used by the placenta for oestrogen synthesis. Tissue and cell culture techniques, together with improved steroid assays, revealed that the adrenal fetal zone is the primary source of DHEA-S. In recent years, the function of the human and rhesus monkey fetal adrenal cortical zones has been reexamined by assessing the localization and ontogeny of steroidogenic enzyme expression.

The primate fetal adrenal cortex is composed of two functionally distinct zones:

- a. the fetal zone, which throughout gestation expresses little or no 3  $\beta$ HSD activity and so primarily produces DHEA-S, with a reduced ability to produce cortisol. It constitutes about 80% of the weight of the adrenal gland at birth (Johannisson, 1979; Seron-Ferre and Jaffe, 1981).



- b. the definitive zone, which is the likely site of glucocorticoid synthesis.

Although cortisol emanating from the fetal adrenal cortex promotes fetal maturation in primates, its role in the regulation of primate parturition, is unclear (Mesiano and Jaffe, 1997). The fetus contributes significantly to its circulating cortisol levels and is a net exporter of cortisol (Beitins *et al.*, 1973). Data from a study of maternal injection of radio-labeled cortisol and cortisone, showed that in the region of 25% of fetal cortisol and approaching 90% of fetal cortisone originate from maternal cortisol (Beitins *et al.*, 1973). Another study found that 85% of cortisol is converted to cortisone in its passage through the placenta (Campbell and Murphy, 1977). It is thought that this process exists to protect the fetus from high levels of maternal cortisol, allowing the fetus to develop its independent HPA axis (Dancis *et al.*, 1978).

#### 2.5.4.2 Cortisol and cortisone

Two isoforms of 11 $\beta$ -hydroxysteroid dehydrogenase (11 $\beta$ -HSD) catalyse the interconversion of active cortisol and inactive cortisone;

**Cortisol** is converted to **Cortisone** by 11 $\beta$ Hydroxysteroid type 2

**Cortisone** is converted to **Cortisol** by 11 $\beta$ Hydroxysteroid type 1

Using a reverse transcriptase, polymerase chain reaction approach, one study group failed to detect 11  $\beta$ -HSD1 mRNA in any human mid-gestational fetal tissues. In contrast 11  $\beta$ -HSD2 mRNA was present in fetal lung, adrenal, colon and kidney (Stewart *et al.*, 1995).

#### 2.5.4.3 *Postnatal changes in cortisol and cortisone*

In the fetal circulation, cortisone predominates over cortisol with a F:E (cortisol:cortisone) ratio of about 0.3 (Nahoul *et al.*, 1988). The F:E ratio rises to >1 in term infants after birth (Kojima *et al.*, 1981; Sippell *et al.*, 1978a). Levels of cortisone have been found to remain constant in preterm and term infants after birth, indicating that changes in F:E ratio are largely due to changes in cortisol concentration (Nomura, 1997). In a group of preterm infants born at gestational ages 24-31 weeks, over the first 3 weeks of life, F:E ratios were also found to be similar to those reported in term infants (Midgley *et al.*, 2001). In a study following infants beyond the first 2 months of life, a relatively high level of cortisone, however, has been reported to remain in premature infants for a longer time than in control term infants. (Fujitaka *et al.*, 1997).

#### 2.5.5 Cord blood cortisol levels

A review of the data for fetal cortisol levels at delivery suggested that the mode of delivery and anaesthetic used influenced levels, as values obtained at elective caesarean section were lower than those after vaginal delivery. Values in premature infants were only about half those of mature infants and lower in those who went on to develop respiratory distress syndrome (Murphy, 1983). In a study of cord serum at birth, there appeared to be a rise in the fetal level of cortisol with gestational age in late pregnancy, whether or not labour took place. This rise was steepest immediately before the normal time of onset of labour and could not be attributed to the stress associated with labour (Murphy, 1982).

#### 2.5.6 Correlation with clinical state

Cortisol plays a pivotal role in the human body's adaptation to physiological and physical stress. Absence of a functioning adrenal gland leads to certain death. Adrenal insufficiency in older adults and children, when compounded by illness also results in severe illness or death. The issue of adrenocortical function and its relationship to illness severity has been

addressed in the adult ITU setting. Random serum cortisol levels have been found to correlate positively with illness severity. Those with the highest random serum cortisol levels (greater than 1655 nmol L<sup>-1</sup>) had the greatest mortality, while those with lower random cortisol levels that stimulated to more than 496 nmol L<sup>-1</sup> after ACTH injection had improved outcomes. The authors suggested that, if adrenocortical insufficiency is suspected, the ACTH test should be performed since low random cortisol levels (even to 138 nmol L<sup>-1</sup>) were not diagnostic of adrenal insufficiency (Jurney *et al.*, 1987).

There is no consensus about the relationship between illness severity and neonatal cortisol levels. The reasons may be several-fold, as outlined at the beginning of this chapter. A detailed review of the literature regarding illness severity and plasma cortisol levels can be found in appendix 1. The studies are summarised in table 2-4. It can be seen that while numerous studies have failed to demonstrate a positive relationship between illness-severity, several recent, well designed studies with good subject numbers, have done so.

#### 2.5.7 Variability/ pulsatility

In the healthy HPA system in the adult and child, pulsile cortisol secretion is characteristic.

To define the pattern of plasma cortisol levels in 14 ELBW infants, Jett et al obtained blood specimens every 20 min over a 6-hour period at 4-6 days PNA. Although cortisol levels in the 14 infants ranged from 55-1503 nmol L<sup>-1</sup>, each infant's cortisol levels varied little from his or her own mean cortisol level. The SDs calculated from each infant's mean cortisol level were small. Cluster analysis was applied to the data: only 0.6 cortisol pulses/infant 6-hour period were detected. Each infant's plasma cortisol levels were plotted against time, and regression analysis was performed. The data indicated that ELBW infants showed little variability in their plasma cortisol levels over time. A single random measurement might

therefore provide an adequate reflection of the adrenal status of the ELBW infant (Jett *et al.*, 1997). Single random plasma cortisol levels are used in the adult ITU setting. Hanna *et al.* also suggests that a single random sample is adequate to indicate adrenal activity (Hanna *et al.*, 1997). This finding was also found by Doerr *et al.* who described less variation in cortisol levels in preterm infants compared to term infants (Doerr *et al.*, 1988).

Metzger found that overall production rates of cortisol were similar in preterm and term infants and comparable to those of adults and children, although the amplitude of pulses were lower, and length of pulses longer in the preterm infants. Several studies have found that cortisol appears to be secreted in pulses every 80 minutes in preterm infants (Arnold *et al.*, 1998; de Zegher *et al.*, 1994; Metzger *et al.*, 1993), and that pulsatility may be affected by exposure to antenatal steroids (Arnold *et al.*, 1998). Metzger found the half-life of cortisol to be 45-56 mins compared with 63 mins in children, and 73 mins in adults (Metzger *et al.*, 1993). In a study of infants > 34 weeks GA, on the first day of life, the median serum cortisol half-life was found to be 60 minutes (de Zegher *et al.*, 1994).

#### 2.5.8 Free levels and CBG

Rokicki *et al.* estimated that 33% of total plasma cortisol is free in well preterm infants in the first week of life (Rokicki *et al.*, 1990). (In healthy adults, free cortisol is 10-15 % of total plasma cortisol). This has been applied by authors such as Hanna *et al.* to estimate free plasma cortisol (Hanna *et al.*, 1997). Doing this, however does not take account of the fact that after saturation of plasma binding sites, the concentration of free cortisol will rise steeply, and so represent more than 33%. It also does not account for the variable levels of CBG in preterm (and sick) infants.

In defining total plasma cortisol levels that constitute adrenal insufficiency, the CBG should be considered, as it is the free cortisol that is biologically active, and involved in feedback

inhibition. CBG levels have been found to be lower and more variable in preterm infants than term and adult patients (Hanna *et al.*, 1997).

CBG levels range from 13-26 mg ml<sup>-1</sup> in preterm newborn infants (Arnold *et al.*, 1998; Kari *et al.*, 1996; Metzger *et al.*, 1993; Scott and Watterberg, 1995). Metzger calculated that the infants had a maximum binding capacity of about 260 nmol L<sup>-1</sup> (1:1 binding of cortisol with CBG, and MW of 52 000 for CBG (Metzger *et al.*, 1993). Arnold found no difference in levels between those treated and those not treated with antenatal steroids. Also, there was no significant correlation between serum total cortisol and CBG levels, or birth weight, and, in agreement with other authors (Scott and Watterberg, 1995; Korte *et al.*, 1996), low CBG levels did not explain low serum total cortisol levels found in some very sick infants (Hanna *et al.*, 1997). In another study, 61 infants had samples analysed for CBG. Levels were lower (16 mg L<sup>-1</sup>) than for children and adults (19-45 mg L<sup>-1</sup>), and there was no significant change with PNA (Scott and Watterberg, 1995). In contrast, Kari *et al.* found that CBG levels did correlate with PNA, but not GA (Kari *et al.*, 1996). Hanna *et al.* found that CBG levels are lower in ELBW infants than in term infants (Hanna *et al.*, 1997).

## 2.5.9 Significance of actual plasma cortisol values: thresholds and definitions

### 2.5.9.1 Adults

In acutely ill adults, a single total plasma cortisol value is thought to be sufficient to make a (presumptive) diagnosis of adrenal insufficiency:

136-360 nmol L<sup>-1</sup> = probable adrenal insufficiency

<136 nmol L<sup>-1</sup> = definite insufficiency

In well adults, adrenal insufficiency = morning cortisol <83 nmol L<sup>-1</sup> (Grinspoon and Biller, 1994)

A peak of 550 nmol L<sup>-1</sup> after ACTH stimulation is the lower limit of normal to describe an adequate adrenocortical response (Karlsson *et al.*, 1999).

#### 2.5.9.2 Older children

In older well children, morning total plasma cortisol levels of about 200 nmol L<sup>-1</sup> would be expected (Sippell *et al.*, 1980)

#### 2.5.9.3 Well preterm infants

Alkalay (Alkalay *et al.*, 1996) performed a meta-analysis of several papers: (Kanarek *et al.*, 1988; Kuhn *et al.*, 1991; Metzger *et al.*, 1993; Thomas *et al.*, 1986; Wiener *et al.*, 1987) with 128 measures from 86 well preterm infants and concluded that: levels > 138 nmol L<sup>-1</sup> were normal in well preterm infants > 12 days old.

Korte *et al* gave a definition of 'low' as <414 nmol L<sup>-1</sup> on day 2 (Korte *et al.*, 1996).

Merz considered baseline morning total plasma cortisol values of >138 nmol L<sup>-1</sup> to be normal.

<83 nmol L<sup>-1</sup> was considered to be very abnormal. (Merz *et al.*, 1998)

#### 2.5.9.4 Stressed preterm infants

In the same review (Alkalay *et al.*, 1996), the following conclusions were also drawn:

Following dexamethasone therapy, a random sample with cortisol >360 nmol L<sup>-1</sup> indicates adequate HPA activity.

Cortisol 360 nmol L<sup>-1</sup> = 3x median cortisol in well non-stressed preterm infants

Cortisol <360 nmol L<sup>-1</sup> in a stressed infant warrants evaluation of adrenal function.

#### 2.5.9.5 ACTH stimulated values in preterm infants

Hingre (Hingre *et al.*, 1994) and Wilson (Wilson *et al.*, 1988) suggest post ACTH stimulation, cortisol lower limit of normal should be 360 nmol L<sup>-1</sup>.

Watterberg and Scott defined adequate response to ACTH as being a rise in cortisol of >250 nmol L<sup>-1</sup> (Watterberg and Scott, 1995)

#### 2.5.10 Papers with total plasma cortisol levels $>1000 \text{ nmol L}^{-1}$

A review of the range of plasma cortisol levels in the literature reveals that many studies have produced results with values  $> 1000 \text{ nmol L}^{-1}$ . This suggests that some infants are able to mount a substantial cortisol response to some aspect of their clinical condition. See table 2-4 and appendix 2.

#### 2.5.11 Suggested adrenal insufficiency

Despite the papers above that describe some very high cortisol values, there is a large body of evidence to suggest that at least a subgroup of preterm and or sick newborn infants demonstrate a degree of adrenocortical insufficiency. Some very preterm neonates admitted to the neonatal intensive care unit show circulatory and respiratory problems that improve after administration of steroids. It is unclear whether these symptoms could be caused by adrenal insufficiency. Al Saedi et al found low levels of cortisol in well infants who did not show signs of insufficiency (al Saedi *et al.*, 1995) and Fujitaka et al argues that low levels of cortisol may not indicate adrenal insufficiency but simply the rapid conversion of cortisol to cortisone (Fujitaka *et al.*, 1997).

See appendix 3 and table 2-4 for details of the relevant studies.

#### 2.5.12 Possible causes of adrenal insufficiency

During the first two weeks of life rat pups show a markedly reduced adrenocortical response to stress, and this period of adrenocortical quiescence has been termed the 'stress non-responsive period' (SNRP). The adaptive value of the SNRP can be understood in terms of the effects of glucocorticoids on CNS development: excessively high or low corticoid levels are associated with abnormal neurological and behavioural development. Researchers have attempted to explain adrenocortical activity during this period in terms of the unique pattern of glucocorticoid-receptor concentrations that exist in the brain and pituitary of the neonatal rat. This pattern of receptor concentrations results in a negative-feedback condition at the

level of the brain and pituitary that ensures the low, stable corticoid levels that appear to be optimal for neuronal development in glucocorticoid-sensitive brain regions (Sapolsky and Meaney, 1986)

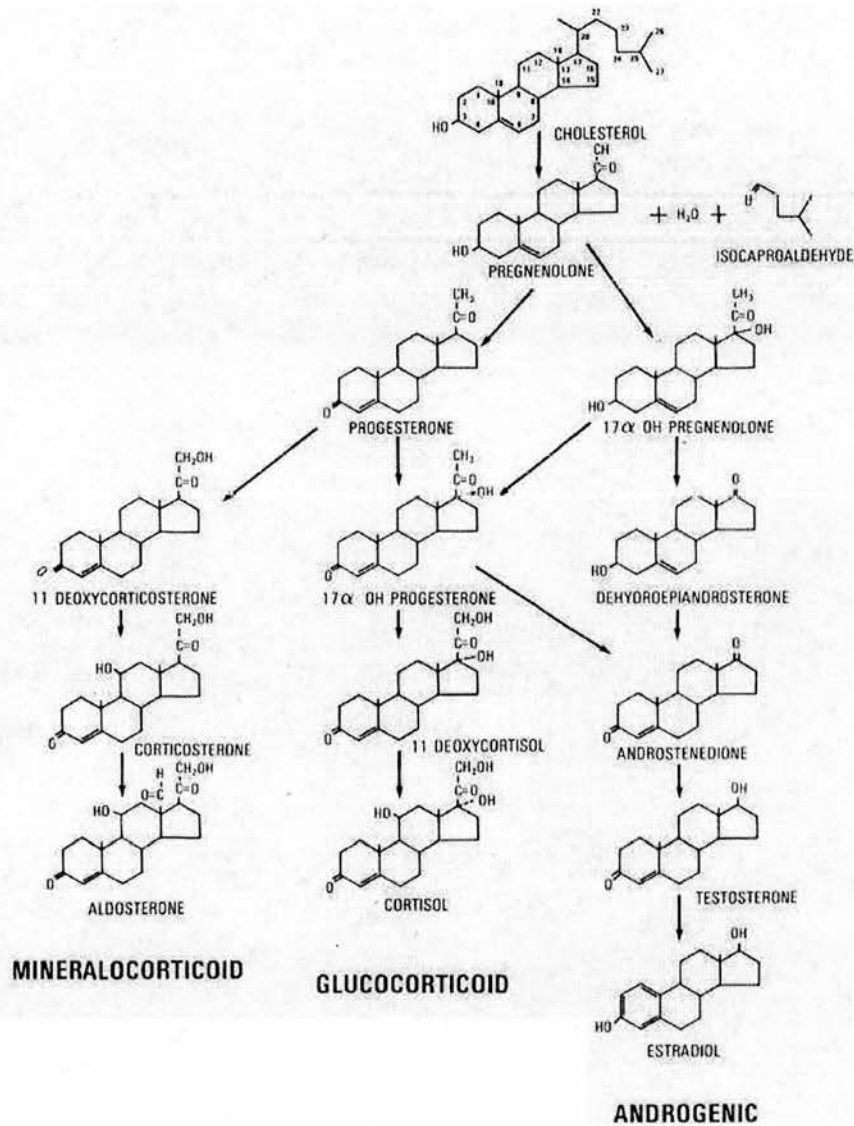
#### 2.5.12.1 *Reduced enzyme activity*

Several studies have produced data that suggest, in preterm and sick infants, maturation dependant reduced activity of 11 $\beta$ -hydroxylase (Hingre *et al.*, 1994; Korte *et al.*, 1996; Lee *et al.*, 1989; Nomura, 1997). Reduced activity of 3  $\beta$ -hydroxysteroid dehydrogenase may also be present (Hingre *et al.*, 1994). The activity of 21-hydroxylase may be reduced; higher baseline levels of 17-hydroxyprogesterone in preterm sick infants compared to term infants have been found by several authors (al Saedi *et al.*, 1995; Huysman *et al.*, 2000; Murphy *et al.*, 1983; Thomas *et al.*, 1986; Wallace *et al.*, 1987; Murphy *et al.*, 1983).

Fujitaka, however, argues that the findings of raised steroid precursors may be due to antibody cross reactivity in immuno-assays leading to falsely elevated levels (Fujitaka *et al.*, 1997). Using HPLC, he did not reproduce the findings. See appendix 4 for a more detailed description of the above studies. Fig 2-4 shows the biochemical pathways discussed.



Figure 2-4 Pathways of steroid biosynthesis.



1.  $3\beta$  Hydroxysteroid dehydrogenase catalyses the conversion of  $17\alpha$  OH Pregnenolone to  $17\alpha$  OH Progesterone.
2. 21 hydroxylase catalyses the conversion of  $17\alpha$  OH Progesterone to 11 deoxycortisol.
3.  $11\beta$  hydroxylase catalyses the conversion of 11 deoxycortisol to cortisol

### 2.5.12.2 Immature HPA regulation

The data in this area are not extensive, and the proposed theories of the nature of a hyporesponsive HPA axis conflicting:

Hanna et al found a 'normal cortisol' response to CRH and ACTH (comparable to older children) in infants born as early as 26 weeks GA (but used high doses of stimulating hormones) and concluded that either the brain of the preterm sick infant was unable to recognise stress, or the hypothalamus was unable to secrete CRH (Hanna *et al.*, 1993).

However, Ng et al showed a high ACTH response to CRH in ventilated preterm infants, without an increase in cortisol levels, suggesting that the pituitary is more mature and functional than the adrenal in preterm infants (Ng *et al.*, 1997c).

Korte et al suggest that there appears to be a dissociation of ACTH and cortisol levels in sick preterm infants, which is similar to data from adult ITU patients. They suggest unresponsiveness at the adrenal level, with many very low birth weight infants having 'low' cortisol ( $<414 \text{ nmol L}^{-1}$ ) concentrations and unable to mount a cortisol response to physiologic doses ( $0.1 \text{ } \mu\text{g kg}^{-1}$ ) of 1-24ACTH (Korte *et al.*, 1996).

### 2.5.13 Changes in cortisol levels with postnatal age.

(see appendix 5)

In general, most studies do not separate sick from well infants in their analysis of trend in cortisol levels over time. Preterm infants are usually sickest during the first days or week or two of life. This is likely to make these earlier values higher compared to later weeks. In both well and sick infants, the response to the stress of labour and delivery is also evident in cortisol levels for the first 48 hours. Neonatal cortisol values after 48 hours do not show any correlation with the type of delivery (Kauppila *et al.*, 1978).

In term infants, cortisol is elevated in comparison with later infancy for about the first week of life. There may be a "glucocorticoid dip" in cortisol 2 and 12 h after birth (Kojima *et al.*, 1981; Sippell *et al.*, 1978b).

In preterm infants, levels are highest in the first 2 days of life (Kraiem *et al.*, 1985; Watterberg *et al.*, 2000). Several authors have described a decline in cortisol levels over the first 2 weeks of life and lower levels appear to be sustained for at least 2 weeks after this (Metzger *et al.*, 1993; Economou *et al.*, 1993; Kanarek *et al.*, 1988; Kauppila *et al.*, 1978; Noguchi and Reynolds, 1978; Banks *et al.*, 2001a)

A fall in cortisol levels over the first 2-3 months of life in preterm and term infants has been described (Arnold *et al.*, 1997; Noguchi and Reynolds, 1978; Rokicki *et al.*, 1990; Wittekind *et al.*, 1993).

However, several studies have failed to demonstrated a relationship between postnatal age and cortisol levels after the first 48 hours in the first weeks of life, (Arnold *et al.*, 1998; Kraiem *et al.*, 1985; Lee *et al.*, 1989; Metzger *et al.*, 1993; Ng *et al.*, 1997b; Watterberg *et al.*, 2000; Bettendorf *et al.*, 1998).

#### 2.5.14 Changes in cortisol levels with gestational age

The relevant studies are described in more detail in appendix 6 and summarised in table 2-4.

When examining the effect of gestational age on cortisol levels, it is logical to account for the potential effect of illness stress on cortisol levels. Preterm infants tend to be sicker than more mature infants. In addressing this question or commenting on this relationship, as with postnatal age, many authors do not differentiate between sick and well infants, or do not perform appropriate analysis to account for degree of illness. Most studies have found preterm infants to have either similar or higher cortisol levels compared to term infants. As outlined above, though, there may be a subgroup of preterm infants who manifest a degree of adrenal insufficiency.

##### *2.5.14.1 Umbilical cord blood levels; 'lower gestation: lower cortisol'*

Maturation dependent cortisol concentrations without the effects of the stress of neonatal illness may be indicated by umbilical cord blood levels. In one study, levels of cortisol were determined in umbilical cord serum in the presence and absence of labour at various gestational ages. Without labour, by 35 to 36 weeks, the mean levels had risen from lower levels in the second trimester and there was a further increase between 37.5 and 40 weeks. Data obtained at vaginal delivery after the spontaneous onset of labour over the period of 26 to 40 weeks were more variable but showed a similar pattern. Thus, there appears to be a rise in the fetal level of cortisol with gestational age in late pregnancy, whether or not labour takes place. This rise is steepest immediately before the normal time of onset of labour and cannot be attributed to the stress associated with labour (Murphy, 1982). These findings have been reproduced by Kari who also found that gestational age correlated with the umbilical cord concentrations of total cortisol and free cortisol (Kari *et al.*, 1996).

#### 2.5.15 Effect of antenatal steroids on neonatal cortisol levels

The literature on the effect of antenatal steroid therapy on the HPA axis in the newborn period is conflicting. Overall, if suppression occurs, it would seem that it is transient, probably resolving within the first few days of life. See appendix 7 and table 2-4 for more details of the relevant studies.

#### 2.5.16 Effect of postnatal steroids on neonatal cortisol levels

ACTH tests have been used widely to attempt to detect adrenal suppression following steroid treatment. Many of the results are conflicting. This may be because the dose of ACTH differs from study to study, as does the timing of stimulated samples. Korte et al suggest that many studies using high doses of ACTH instead of low, physiological doses masks the presence of functional HPA suppression (Korte *et al.*, 1996). Karlsson found that the peak of plasma after a low dose ACTH test occurred at 30-40 minutes, while after a standard dose test occurred after 60-120 minutes (Karlsson *et al.*, 1999).

Most investigators have demonstrated a degree of adrenal suppression in infants with CLD treated with dexamethasone. Longer courses of dexamethasone tend to induce more suppression than short (Alkalay *et al.*, 1990; Bettendorf *et al.*, 1998; Bouchier and Weston, 1997; Cronin *et al.*, 1993; Kari *et al.*, 1993; Kari *et al.*, 1996; Merz *et al.*, 1998; Ng *et al.*, 1989; Rizvi *et al.*, 1992; Scott *et al.*, 1997; Wilson *et al.*, 1988).

Some authors, however have failed to demonstrate evidence of significant suppression following dexamethasone therapy (Brundage *et al.*, 1992; Cole *et al.*, 1999; Giep *et al.*, 1996; Rennie *et al.*, 1989; Wittekind *et al.*, 1993).

### 2.5.17 Effect of opiates on neonatal cortisol levels

Baseline median plasma cortisol in a group of ventilated infants requiring sedation, median age 15 hours, was 364 nmol L<sup>-1</sup>. This fell significantly to median 162 nmol L<sup>-1</sup>, 6 hours after opiate sedation was commenced (Barker and Rutter, 1996).

Vaughn et al found that cortisol mirrored the analgesic effects of opiates in term infants in the post-operative period (Vaughn PR, 1996b).

Pokela studied the effects of analgesia on plasma beta-endorphin, serum cortisol and blood glucose responses in 20 distressed, mechanically ventilated neonates during the first 3 days of life. Cortisol values decreased significantly 2 h after analgesia. The results indicated that the stress response in the distressed neonates with cardiorespiratory problems, as assessed by cortisol, beta-endorphin, and blood glucose, was attenuated by opioid medication (Pokela, 1993).

### 2.5.18 Association with growth retardation/foetal distress/stress

In a study of infants with GA < 30 weeks during the first two weeks of life, small for gestational age infants (n = 8) had significantly higher cortisol values (median 357 nmol L<sup>-1</sup>) than AGA infants (median 199 nmol L<sup>-1</sup>) (Heckmann *et al.*, 1999).

Increased cortisol levels in cord plasma have been associated with spontaneous vaginal delivery but not with chronic fetal distress (Ruth *et al.*, 1993). In agreement, Gutai found no difference in cortisol levels between normal infants and infants exposed to antenatal stress in the form of maternal febrile illness, drug addiction or diabetes (Gutai *et al.*, 1972).

### 2.5.19 Birth Weight

Some authors have found a significant relationship between BW and cortisol levels:.

In infants with BW <1500, 24-33 weeks, AGA, and ventilated, the cortisol response to ACTH increased with increasing birth weight (Watterberg and Scott, 1995).

In a study of 61 infants <32 wks and < 1500g, serum cortisol concentrations on day 7 were significantly higher in infants of greater birth weight. (Ng *et al.*, 1997c).

Birth weight was required as a cofactor in analysis of cortisol levels measured on days 5 and 7 in patients who developed CLD (Watterberg and Scott, 1995).

However, no significant relationship was found between salivary cortisol levels and gestational age, postnatal age or birth weight in either full-term or preterm infants, who were well and stable (Bettendorf *et al.*, 1998). Similarly, Arnold *et al* have found no association between mean cortisol over 6 hours and BW (Arnold *et al.*, 1998).

### 2.5.20 Hypotension

Dexamethasone has been found to be effective in the management of severe arterial hypotension in some (5/8) preterm infants not responding to standardized treatment, implying that adrenal insufficiency may play a role in the aetiology of hypotension (Gaissmaier and Pohlandt, 1999). Helbock *et al* have reported a group of hypotensive preterm infants who had low cortisol values prior to responding to treatment with corticosteroids (Helbock *et al.*, 1993). However, in a randomised trial of dopamine or hydrocortisone for the treatment of hypotension in preterm infants, there was no difference in pre-treatment basal cortisol or ACTH stimulated cortisol in those who did and those who did not respond to steroids. Dopamine and hydrocortisone were equally effective for the treatment of hypotension (Bourchier and Weston, 1997).

### 2.5.21 Chronic Lung Disease

One study showed that at the end of the first week of life, infants who subsequently developed BPD and prolonged oxygen dependence had significantly lower cortisol secretion in response to ACTH on days 5-7 than infants who recovered without BPD. The authors speculated that these babies may have been unable to secrete adequate amounts of cortisol in a setting of increased stress, leaving them vulnerable to continuing lung injury (Watterberg and Scott, 1995). These findings were backed by a subsequent study that showed infants with lower cortisol values in the first week of life had an increased incidence of PDA, increased lung inflammation, and an increased incidence of chronic lung disease (Watterberg *et al.*, 2000). Infants who failed to resolve their lung disease had significantly lower cortisols than other infants (Scott and Watterberg, 1995).

Korte *et al* found an association between CLD outcome and low basal cortisol (less than 414 nmol L<sup>-1</sup>), but no association between ACTH stimulated cortisol and outcome, leading the authors to suggest that actual and not potential cortisol levels are significant in these infants (Korte *et al.*, 1996).

However, in a study of 314 infants GA 24-32 weeks, basal plasma cortisol concentration during the first week was found to be only a weak predictor for CLD (Banks *et al.*, 2001a). Mertz *et al* found that no association could be found between the synthesis of cortisol and ACTH at the end of the first week of life and the development of chronic lung disease (Merz *et al.*, 1998). Furthermore, neither Ballard *et al*, nor Doer *et al* found an association between early cortisol levels and the subsequent development of CLD (Ballard *et al.*, 1980; Doerr *et al.*, 1988)



### 2.5.22 Cortisol and pain in infants

In normal term infants <1 month old, after the painful stimulus of a venipuncture, plasma cortisol increased significantly, from a mean of 41 nmol L<sup>-1</sup> to 458 nmol L<sup>-1</sup> at 30-60 minutes. (Mantagos *et al.*, 1991).

Total plasma cortisol also increases significantly after circumcision in the newborn period:

1. mean 160 nmol L<sup>-1</sup> to 406 nmol L<sup>-1</sup>, 20 minutes post circumcision (Talbert *et al.*, 1976)
2. mean 221 nmol L<sup>-1</sup> to 662 nmol L<sup>-1</sup> 30 minutes post circumcision (Gunnar *et al.*, 1984)
3. mean 273 nmol L<sup>-1</sup> to 632 nmol L<sup>-1</sup> 30 mins post circumcision (Williamson and Evans, 1986)

### 2.5.23 Summary

While it can be difficult to draw firm conclusions from the literature about neonatal plasma cortisol levels and their associations, it seems reasonable to make the following generalisations:

1. A sizable proportion of preterm and sick infants appear to be capable of producing high or appropriate concentrations of plasma cortisol but a subgroup may manifest cortisol insufficiency.
2. Steroid metabolism and HPA axis regulation in the newborn, preterm or sick infant differ from those of the more mature and less sick infant. The pulsile nature of cortisol production, CBG levels and free cortisol levels are all affected.
3. Gestational age, postnatal age and illness severity appear to interact in a complex, currently poorly understood manner that leads to the general findings of: sicker, younger, less mature infants tending to have higher cortisol levels than less sick, older, more mature infants.
4. Antenatal steroid exposure has short-term, rapidly resolving suppressive effects on neonatal plasma cortisol levels and postnatal steroid therapy has mostly been found to induce a significant degree of HPA suppression that may depend on the length of course of steroid treatment.
5. Newborn infants are capable of mounting a cortisol response to acute painful stimuli and the use of opiates can reduce plasma cortisol levels.
6. Despite the theoretical potential, there appears to be no strong relationship between plasma cortisol levels and hypotension or CLD.

Future studies of cortisol levels in sick and preterm newborn infants may be easier to interpret if more rigorous attention is paid to the following areas:

1. Standardisation of subject characteristics, particularly in relation to GA and PNA
2. Use of validated illness severity scores.
3. Incorporation of 'no-handling' periods +/- distress scores
4. Use of a technique to account for pulsatility/ variation
  - a. Multiple sampling
  - b. Microdialysis
  - c. Diffusion sink
5. Multi-centre studies to increase study numbers
6. Standardisation of sample analysis technique +/- measurement of free or indicator of free, biologically relevant cortisol.

Appendices:

Appendix 1 Studies examining the relationship between cortisol and degree of illness

Appendix 2 Studies with total plasma cortisol values  $> 1000 \text{ nmolL}^{-1}$

Appendix 3 Studies with low total plasma cortisol levels in sick infants

Appendix 4 Studies suggesting reduced enzyme activity as cause for cortisol insufficiency

Appendix 5 Changes in cortisol levels with postnatal age

Appendix 6 Changes in cortisol levels with gestational age

Appendix 7 Effect of antenatal steroids

Author	Year	Aims	Population	nos	age	Freq.	meth od	Sub- group	max	min	Median / mean	Notes
Alkalay (Alkalay <i>et al.</i> , 1990)	1990	HPA function in VLBW infants treated with dex. Metrapone test.	BW 825 GA 25.8	10	33 days	Basal + post met	RIA	Non-suppressed Suppressed			400  82.8	5/10 infants showed suppression after long course of dex.
Anders (Anders <i>et al.</i> , 1970)	1970	Relate cortisol to behavioural state	Healthy newborns	4	1-15 weeks	20X each infant	PBM		552	0	Est 91	Increased cortisol after 20 mins of crying
Arnold (Arnold <i>et al.</i> , 1998)	1998	Effect of antenatal steroids on pulsatility of cortisol secretion	25-33 weeks	26	<9 days 5 day 2 1 <24 hours		RIA		501	15	176 median	Amplitude but not number or length of pulses less in antenatal steroid group. Blood pressure related to frequency of pulses and cortisol production rate.
Arnold (Arnold <i>et al.</i> , 1997)	1997	Normal values for cortisol and 17OHP in 1 <sup>st</sup> 16 weeks of life	Median 28weeks GA Well and sick	26	1-16 weeks		RIA		596	28	88 med	Decreasing cortisol + 17OHP over 1-8 weeks. No correlation of cortisol with clinical state. Higher 17OHP in sicker infants at 5-8 weeksPNA. Higher 17OHP in preterm than in term.
Banks (Banks <i>et al.</i> , 2001a)	2001	Explore relationship between CLD and basal cortisol levels	24-32	314	1 <sup>st</sup> 28 days		RIA	Birth 24hrs 14-28 days			85.5 535.2 162.8	No association with GA in 1 <sup>st</sup> week of life. CRIB assoc with cortisol on day 1, 3, 7, 1 <sup>st</sup> week cortisol is weak predictor of CLD.

Author	Year	Aims	Population	nos	age	Freq.	meth od	Sub- group	max	min	Median / mean	Notes
Barker (Barker and Rutter, 1996)	1996	Examine relationship between stress response and severity of illness	Ventilated Preterm 28 weeks med	47	15 hours	Prior to and 6 hrs post diam orph.	?		7799	26	Med 364 prior to diamorp h	Stress response proportional to a:A ratio. Catecholamine response assoc with worse outcome. Significant decrease in cortisol post diamorph.
Bourchier (Bourchier and Weston, 1997)	1997	Randomised trial of dopamine vs hydrocortisone for hypotension in preterm infants. ACTH test prior to and 24 hours post Tx	Mean GA 26-27 Hypotensi ve	40	<1day at time of Tx.		IFA	basal stim			500 822	Hydrocortisone effective treatment for hypotension. Data did not infer low cortisol as cause for hypotension. No difference in basal cortisol or ACTH response pre and post treatment.
Calixto (Calixto <i>et al.</i> , 2002)	2000	Saliva related to plasma cortisol pre and post ACTH	26-33 weeks GA	48 infa nts 144 pairs	Day3- 7		RIA	Mean base- line			27.6 saliva 340 plsama	R=0.68 correlation between plasma and saliva.

Author	Year	Aims	Population	nos	age	Freq.	meth od	Sub- group	max	min	Median / mean	Notes
Cole (Cole <i>et al.</i> , 1999)	1999	ACTH after beclamethason e Tx	<1250gr <33 weeks	85	21 days		RIA				151	Lower basal mean in beclamethasone group, but no diff in stimulated cortisol level.
Cronin (Cronin <i>et al.</i> , 1993)	1993	ACTH test after 36 days dex	PCA at test 39+/-1 week	14	Est 70 days		RIA	Basal Stimu lated	543 934	0 117	87 568	Post dex, normal response to ACTH but lower basal cortisol levels. Some levels est >1000nmol/l.
De Zheger (de Zegher <i>et al.</i> , 1994)	1994	TSH and cortisol on 1 <sup>st</sup> day of life	34-41 polycytha emic	9	1 day 4 days	Every 20 mins over 6 hours	RIA		No value s given			Pulsatile secretion.
Economou (Economou <i>et al.</i> , 1993)	1993	Examine cortisol secretion in stressed babies over 1st 4 weeks of life	Term well Term sick Preterm well 33.5 Preterm sick 32	60	Day 1 3 5 10 15 30	0800 1400 2000 0200 hrs	IFA		13 value s > 1000	well sick well sick	143 268 165 360 day 3 means	Preterm well had cortisol > term well. Sick and preterm infants had higher values in the evening. Highest levels were in sick preterm and term infants. General decrease from day 1-15. 2 infants who died had cortisol consistently >1600.4/15 term and 6/15 preterm 'poor responders' with cortisol <220.

Author	Year	Aims	Population	nos	age	Freq.	meth od	Sub- group	max	min	Median / mean	Notes
Fujitaka (Fujitaka <i>et al.</i> , 1997)	1997	Relationship of cortisol :cortisone + changes with prematurity and age	preterms children adults	232 127 88	>4 days <1 month	single	HPL C+ UV				Mean 79.6 < 1 month	In controls over 2 months of age, cortisol>cortisone but prolonged cortisone > cortisol in preterm infants. Under 2 months of age, cortisone > cortisol. Recommends cortisol and cortisone be measured in assessing preterm infants.
Giep (Giep <i>et al.</i> , 1996)	1996	DBPC trial of beclamethason e inhaled for 7 days	Ventilated + failed extubation <1500	19	>2 weeks		RIA	basal			270	No suppression detected. Beclamethasone may enhance extubation
Gunnar (Gunnar <i>et al.</i> , 1985)	1985	Sleep and plasma cortisol during recovery from circumcision	Healthy term	80	2-3 days	30, 90, 120, 240 post circ	RIA		No values			Sleep associated with rapid reduction in cortisol.
Gunnar (Gunnar <i>et al.</i> , 1987)	1987	Relationship between behavior assessment and cortisol	Well and slightly unwell term	60	32-122 hrs	Singl e	RIA		630	66	240 mean	No associations within the group as a whole. Ill infants who had a high cortisol displayed more distress behaviour. Well infants with higher cortisols had better adaptation and more favourable NBAS scores.

Author	Year	Aims	Population	nos	age	Freq.	meth od	Sub- group	max	min	Median / mean	Notes
Gutai (Gutai <i>et al.</i> , 1972)	1972	Normal plasma levels ACTH test in well and sick.	Term	50	1st 3 days	2x/day 2 hrs post ACTH	PBM	Well Stress ed. Basal	361 689	28 28	113 143	Good ACTH response. No diff in basal levels between well and sick but sick infants not that sick.
Guttenberg	1993	Glucocorticoid pathway in ill and well ELBW infants	<1000g	27	3 days	single	RIA+ chro m				228	Sicker babies did not have higher levels, but had higher precursors.
Hanna (Hanna <i>et</i> <i>al.</i> , 1997)	1997	CBG and cortisol and stress in ELBW infants	<28 weeks	31	<8 days		RIA				250	SNAP-PE did not correlate with CBG or cortisol. No correlation between CBG and cortisol. CBG 200nmol/l binding. Lower in preterm than term. Insufficiency in 12/32 infants. Suggests that single sample adequate to assess HPA activity.
Hanna (Hanna <i>et</i> <i>al.</i> , 1993)	1993	Investigate preterm HPA axis, oCRH and ACTH stimulation.	24-31 mean 26	17	2-7 days mean 4.1	CRH test ACTH test		Basal CRH Basal ACTH			394 568 603 882	Normal response of adrenal to ACTH and normal response of pit to CRH. Inappropriately low cortisol may be due to poor brain recognition of stress and CRH production. CBG levels lower than adults, but not related to cortisol levels.
Heckman (Heckmann <i>et al.</i> , 1999)	1999	Reference range for cortisol in well preterm infants	<30 weeks GA. Well, +antenatal steroids	37	<2wk s PNA	Singl e	RIA		562	73	199 median	Non-gaussian distribution, normal if log transform. 8 SGA had higher cortisol. 2 outliers had 1233 and 2050. Negative correlation of cortisol with GA.



Author	Year	Aims	Population	nos	age	Freq.	meth od	Sub- group	max	min	Median / mean	Notes
Heckmann (Heckmann <i>et al.</i> , 2000)	2000	Serum cortisol in ill and well infants	<30 weeks well ill+cats ill	46 25	<2 weeks	random	RIA	Cont Ill Hypot ensive	659 1007 1482 378	58 58 764 41	240 268 1200 110	No diff in cortisol between ill and well. Bimodal cortisol in hypotensive 71% of those with hypotension had low cortisol, but no features distinguished those who did from those who did not.
Hingre (Hingre <i>et al.</i> , 1994)	1994	Levels and pattern of related adrenal steroids	<30 weeks sick, vent, mod- severe illness	25	4 days	ACTH	RIA	Basal Stim			204 504	Similar cortisol levels to term well infants but higher levels of cortisol precursors in sick and preterm infants Post ACTH cortisol lower in sick preterm infants
Hughs (Hughes <i>et al.</i> , 1987)	1987	Look at basal blood spot glucocorticoid concentrations	Sick preterm infants. 24-32 wks	10	3-13 days		RIA		4000		Mean 2000 on day 3-5	4000 in IVH Most > 1000nmol/l. Wide variation. Suggests ITU more stressful than surgery.
Huysman (Huysman <i>et al.</i> , 2000)	2000	Cortisol and ACTH test levels +/- illness and relating cortisol+outco me.	25-29 weeks	21	Day4	Basal 30 60 120 mins	RIA				277 whole group 694 stim	Basal 17OHP correlated with SNAP. High SNAP assoc with low cortisol response to ACTH. No relationship between basal cortisol and SNAP. Adverse outcome associated with lower 60 min cortisol post ACTH.
Jett (Jett <i>et al.</i> , 1997)	1997	Describe pulsatile nature of cortisol secretion	<1000g <28 wks	14	4-6 days PNA		RIA		1503	55		Little variability. Discusses literature on cortisol insufficiency in ELBW infants. Suggests single sample adequate. Cortisol did not correlate with SNAP score or clinical state.

Author	Year	Aims	Population	nos	age	Freq.	meth od	Sub- group	max	min	Median / mean	Notes
Kari (Kari <i>et al.</i> , 1993)	1993	RDBPC trial of dex for BPD	Mean GA 27 weeks Vent dep	41	10 days 17 days 28 days		RIA	10 d 17 d 28 d	640 704 1106 place bo	123 160 137 place ebo		Short but significant suppression after 1 week dex
Kari (Kari <i>et al.</i> , 1996)	1996	Effect of dex and GA on plasma cortisol, DHEAS and binding globulins. ACTH test.	26+/-2 ill + controls +term	36 (23 dex)	21 days	ACTH test after 1 week of place bo	RIA	Basal Stim Place bo gp			176 817	Suggests hypocorticism. in preterm infants Most in first week had <200nmol/l. Dex reduces cortisol response which resolves by 1 week. Umbilical cord cortisol correlated with GA. Some values > 1000nmol/l.
Kaupilla (Kauppila <i>et al.</i> , 1978)	1978	Assess GA and neonatal factors' influence on cord and neonatal cortisol values.	Well ill GA:	92 infants	2d 4d 6d Cordially, 60min		RIA + lipide x chro mato graph y					Lower cord cortisol in elective compared with emergency c-section and SVD. Neonatal values not effected by delivery or GA. RDS assoc with higher cortisol. AN steroids assoc with lower cortisol at 60mins only.
Kojima (Kojima <i>et al.</i> , 1981)	1981	Serum steroid levels at birth and early neonatal period.	Term . No details	20	1-10 days			Day 3	164	18.7	103	Cord levels higher than day 1 then increase over 5 days then decrease.

Author	Year	Aims	Population	nos	age	Freq.	meth od	Sub- group	max	min	Median / mean	Notes
Korte (Korte <i>et al.</i> , 1996)	1996	Low dose ACTH test. Relate adrenocortical function to neonatal outcome.	<32 weeks <1500gr vent +UAC	67	2-3 days	60 mins peak	RIA					Many LBW infants had low cortisol<414. Suggests low cortisol may contribute to BPD. 11 $\beta$ Hydroxylase may limit cortisol production.
Kraiem (Kraiem <i>et al.</i> , 1985)	1985	Describe normal levels in term and preterm infants	Term 32+/- 2		<48 hours 3 days 10 days		RIA	Term Preterm 3-10 days			262 218	SVD cortisol > c-section
Lee (Lee <i>et al.</i> , 1989)	1989	Evaluate serum adrenal steroid levels in preterm infants	31-35 GA mild RDS	13 well 9 ill	2-5 days	spot	RIA	Well  Ventil ated Mean			190  165	No diff between sick and healthy preterms. Preterm levels similar to healthy term levels. Elevated cortisol precursors. Suggests 11 $\beta$ hydroxylase deficiency.
Mantagos (Mantagos <i>et al.</i> , 1991)	1991	Evaluate stress test for HPA axis in 1 <sup>st</sup> 6 months of life	Term normals	33	<1 month 1-3 mo 3-6 mo		RIA	<1 mo 1-3 mo 3-6 mo	458 392 455 post vene	41 72 97 bas al		Significant increase in response to venepuncture in when HPA intact. One value 1377.

Author	Year	Aims	Population	nos	age	Freq.	meth od	Sub- group	max	min	Median / mean	Notes
Mantagos (Mantagos <i>et al.</i> , 1998)	1998	Diurnal variation	term	70 40< 1 month	0-6 months	vene	RIA	Am Pm week1			159 129 means	Diurnal rhythm at 2-3 months No rhythm in 1-4 weeks group.
Merz (Merz <i>et al.</i> , 1998)	1998	Relate CLD to adrenal insufficiency. CRH to examine dex effect.	Vent <1250 GA 24-30	34	4-7 days or 2 day post dex.		RIA	Basal	817	145	346	At 1 week: no assoc with ACTH and cortisol response to CRH and CLD 12/34 had basal cortisol<138 8/34 had basal cortisol<83. No difference in perinatal data between suppressed and non-suppressed infants.
Metzger (Metzger <i>et al.</i> , 1993)	1993	Pulsatile secretion and clearance of cortisol	Preterm Term	5 5	38-94 days					Pre m Term m	350 277	No diff in mean cortisol concentrations between term and preterm infants. Some differences in pulsatile nature and secretory rates. Preterm had lower amplitude and longer bursts.
Midgley (Midgley <i>et al.</i> , 2001)	2001	To examine changes in F:E ratio after birth + urine metabolites	24-31GA	22	2hrs-several weeks		RIA	D1 D3 D7	2036 1654 1400	1 66 98	380 234 280	Fetal FE ratio does not persist after birth

Author	Year	Aims	Population	nos	age	Freq.	meth od	Sub- group	max	min	Median / mean	Notes
Murphy (Murphy <i>et al.</i> , 1983)	1983	Plasma 17OHP in ill newborns	Sick preterm and term Healthy term	47 53	2-10 days		RIA					ill preterm>healthy preterm>ill term>healthy term.
Ng (Ng <i>et al.</i> , 1997b)	1997	CRH response in preterm infants	<32 weeks	14	Day7 Day14		RIA	basal stim			396 647	No difference between 7 and 14 days Good response to CRH
Ng (Ng <i>et al.</i> , 1997c)	1997	CRH test to evaluate effect of AN steroids	<32 weeks	61	D7 D14		RIA					No significant suppression in those exposed to AN steroids. Day 7 cortisol higher in >GA, >BW, lower apgar score, SVD. Day 14 cortisol higher in ventilated compared to non-ventilated.
Ng (Ng <i>et al.</i> , 2001)	2000	Examine levels of leptin and metabolic hormones in preterm infants	29.6 weeks median 29.6 weeks ga	43		daily	RIA	Day1 Day4-5	311 322	89 99	145 201	Cortisol higher in preterm infants on day 4-5 and following instrumental delivery. Leptin regulation linked to HPA axis.
Ng (Ng <i>et al.</i> , 1997a)	1997	CRH test prior to and post 3 week tapering dose of dex for BPD.	VLBW 3 weeks dex	23	0 wk3,4,7,28 days mean		RIA	Basal Stim			427 619	Severe suppression of the adrenal after dex, but recovery 4 weeks after end of the course. Pit recovers before adrenal.

Author	Year	Aims	Population	nos	age	Freq.	meth od	Sub- group	max	min	Median / mean	Notes
NG (Ng <i>et al.</i> , 1999)	1999	Look at effect of 8 courses of antenatal steroids with CRH test	24-34 weeks	14	7 days		RIA	Basal Stim			283 552	No difference between stimulated cortisol between days. Concluded minor if any suppression. Similar response to ACTH as older children and adults.
				12	14 days							
Noguchi (Noguchi and Reynolds, 1978)	1978	ACTH tests	Preterm Well 28-34	15	5-10 days 27-31 days		RIA+ ether extra ction	5-10 days 1 month			152 66 basal	Cortisol and DHAS significantly higher at 5-10 days than 27-31 days. Significantly higher response to ACTH at 27-31 days compared to 5-10 days.
Nomura (Nomura, 1997)	1997	HPLC to examine precursors in pathway in preterm infants	Mean 987gr, 27.4	33	>4 days	94 samples	HPLC	1st month n=25			110	Preterm cortisol > term. 21 hydroxylase 11 $\beta$ hydroxylase 18 hydroxylase all decreased activity inferred.
Onishi (Onishi <i>et al.</i> , 1983)	1983	Postnatal development of circadian rhythm	Term +	64	1 month -15 years	6hrly	HPLC				Mean est 220 1-5mo	Circadian rhythm at 6 months
Parker (Parker, Jr. <i>et al.</i> , 1994)	1994	Abnormal steroidogenesis in growth retarded newborn infants.	Well term IUGR	47 47	birth			IUGR Cont	1415	146	455 408	DHEAS lower in IUGR than controls. IUGR cortisol higher than controls.

Author	Year	Aims	Population	nos	age	Freq.	meth od	Sub- group	max	min	Median / mean	Notes
Pokela (Pokela, 1994b)	1994	DBPCRT morphine to reduce hypoxia and clinical instability in et suction.	Ventilated ,	84	<1 week	Basal , 2, 12, 24 hrs post suct	RIA		3180	60	420 med	No significant diff in cortisol between groups, but decrease in hypoxia in morph group. Higher cortisol < 1 day old. Used CHEOPS and FACES scores.
Procianoy (Procianoy and Cecin, 1985)	1985	Mixed cord blood cortisol and DHEAS after preterm delivery		169	birth		RIA	VD C- SEC			364 160	Cortisol levels proportionate to DHEAS levels. Cortisol higher after VD than C-SEC.
Rennie (Rennie <i>et</i> <i>al.</i> , 1989)	1989	Effect of 13 day dex course on ACTH response	GA 26 mean	12	22 days at start of dex	5-28 days post dex	RIA	Basal Stim	131 229	83 77	98 554	Significant response to ACTH pre and post dex. No change with PNA or in dex treatment
Reynolds (Reynolds, 1973)	1973	Cortisol and related steroids in preterm infants with RDS	Fatal Non-fatal Benign RDS	15 13 16	1 <sup>st</sup> week		Saph edex chro mato graph y, CB	D3 benign D3 non- fatal D8 fatal			267 402 513	High cortisol levels in fatal RDS 1 <sup>st</sup> 3 days of life +>6 days. Some levels >1000. All groups responded to ACTH.
Rizvi (Rizvi <i>et al.</i> , 1992)	1992	CRH test prior to 7 days dex	BPD 27+/-5 weeks	10	7.5 weeks	30 mins peak	RIA	Basal Stim			275 620	Significant suppression at day 7 at site of pit gland. Range 82-2234.

Author	Year	Aims	Population	nos	age	Freq.	meth od	Sub- group	max	min	Median / mean	Notes
Rokicki (Rokicki <i>et al.</i> , 1990)	1990	Total and Free plasma cortisol	Term Preterm SGA	75 88 38	Up to 3 months		Equilibrium dialysis + RIA	Day 3-5 Term Preterm			63 252	Decrease in total and free cortisol over 3 months. Both generally higher in preterm. Term SGA pattern like that of term infants.
Ruth (Ruth <i>et al.</i> , 1993)	1993	CRH + cortisol in cord plasma in relation to age, labour + fetal distress.	Preterm -Healthy -PROM	27 preterm	birth		RIA	SVD c-sect			321 213	No correlation between cord CRH and cord cortisol. High cortisol associated with SVD but not chronic fetal distress
Saedi (al Saedi <i>et al.</i> , 1995)	1995	Reference range for cortisol and 17OH in preterms	Mean 29 weeks Well	39	5 days to 37 weeks PCA	weekl	RIA		680	20	125+/- 107	Low cortisol levels may be normal in the preterm population.
Scott and Waterberg (Scott and Waterberg, 1995)	1995	Effect of GA, PNA and illness on plasma cortisol	24-36 weeks	120	Day 2, 4, 6	Am and pm	RIA		250	8	140	Youngest infants had highest cortisol. High ventilatory requirement had lowest cortisol
Scott (Scott <i>et al.</i> , 1997)	1997	Influence of 5 days dex on ventilation and ACTH test	24-31 GA	15	Pre-dex 8-24 days			Basal Stim			300 750	5 days of dex may be enough to give ventilator independence. Difference in peak cortisol levels between dex and placebo group.



Author	Year	Aims	Population	nos	age	Freq.	meth od	Sub- group	max	min	Median / mean	Notes
Sippell (Sippell <i>et al.</i> , 1978b)	1978	Adrenal steroid hormones from birth to early neonatal period.	Term well	12	Birth- d7		Chro mato graph y and RIA	Birth D4 D7			380 62 60	Significant fall over 6 days following birth.
Sippell (Sippell <i>et al.</i> , 1978c)	1978	Longitudinal study of aldosterone and corticosteroids at birth and after.	Term SVD	12	2, 4, 6, 12, 24 hours 4+7 days		Chro mato graph y and RIA	24 hrs D4 D7			75 159 96	Cortisol elevated for 24 hours then fell.
Stevens (Stevens, 1970)	1970	Examine plasma cortisol in neonatal period	Well term infants	131	1st week				1655	0	Est 275	
Talbert (Talbert <i>et al.</i> , 1976)	1975	Cortisol and cortisone response to circumcision	term	5	<6hrs	Pre+ 20+4 0 mins post circ		Pre Post	551	33	160 405	Significant increase in cortisol post circ.
Thomas (Thomas <i>et al.</i> , 1986)	1986	Response to ACTH in Newborn	28-36 well 28-35 ill 38-41 ill	15 26 11	3-4 days	ACT H test	RIA no Ext		6319 96 646 stimu lated	306 536 372 bas al		No significant difference in basal cortisol or ACTH response between groups. 10% of stressed newborns do not produce basal cortisol. 17OHPr greater in preterms than terms.

Author	Year	Aims	Population	nos	age	Freq.	meth od	Sub- group	max	min	Median / mean	Notes
Watterberg + Scott (Watterberg <i>et al.</i> , 2000)	2000	Links between early adrenal function and resp outcome	<1500 gr	125 infa nts  337 val ues	2-7 days	3X/d ay	RIA	Cld Non- cld			125  168  geometr ic mean	Low cortisol in 1 <sup>st</sup> week associated with CLD, PDA, and increased lung inflammation.
Watterberg and Scott (Watterberg and Scott, 1995)	1995	Evidence of adrenal insufficiency in infants who develop BPD. ACTH at 1 week	<1500 24-33 vent	59	5-7 days	30 min peak	RIA				140 mean	Infants who develop BPD have blunted cortisol response to ACTH. Lower cortisol in infants requiring inotropes.
Weiner (Wiener <i>et al.</i> , 1987)	1987	Normal adrenal steroid levels for diagnosing CAH	Term healthy	16	Day3	Rand om dayti me	RIA		386	47	171	8/16 had <138nmol L <sup>-1</sup>
Williamson (Williamson and Evans, 1986)	1986	RCT of dorsal penile block for circumcision	Term	11bl ock 13 cont rols	24-72 hours	Pre and 30 mins post	?	Contr ols Post circ			273  632	No cortisol response if no circumcision. No sig reduction in cortisol with block.
Wilson (Wilson <i>et al.</i> , 1988)	1988	DBPCT dex and effects	BPD mean 30 weeks	27	3 weeks		RIA	Basal ACT Hstim			226  649	Supression after 7 days of dex but recovery 10 days after end of course. No effect of AN steroids detected.

Author	Year	Aims	Population	nos	age	Freq.	meth od	Sub- group	max	min	Median / mean	Notes
Wittekind (Wittekind <i>et al.</i> , 1993)	1993	Plasma ACTH and cortisol in VLBW in 1 <sup>st</sup> 8 weeks of life and effect of dexamethason e and clinical condition	24-33 weeks, mean 28	25	1			Wk 1			216	Significant decrease week 1 to 8. No correlation between sickness and cortisol.
					2 4 8 post natal weeks			Wk 8			50 medians	Plasma ACTH but not cortisol was decreased in the infants treated with dex.

Table 2-4 Summary of literature on plasma cortisol levels in the newborn period

## 2.6 Illness Severity Scoring

### 2.6.1 Background

For the purpose of the present study, an estimate of illness severity is required at the time of saliva sampling. This is to assess the extent that the acute physiological stress of illness, as opposed to psychological *distress*, might influence salivary cortisol.

Table 2.5 gives an overview of illness severity scoring systems.

A widely accepted definition of severity of illness is the probability of mortality (Knaus *et al.*, 1981; Knaus *et al.*, 1991). Thus most illness severity scores are validated by their ability to predict mortality.

The assessment of illness severity in patients requiring intensive care was first addressed scientifically with the development of the APACHE score for use in adult intensive care units (ITU) (Knaus *et al.*, 1981). This was a score designed for use on admission to ITU. The score has since been refined and modified to the APACHE III version that is now in widespread use (Knaus *et al.*, 1991). This scoring system is used to compare the performance of ITUs, in internal unit audit, as a component of ITU based research and of day-to-day assessment and charting of an individual patient's progress.

A similar score for use in paediatric ITU was developed, taking account of differences in physiology between adult and paediatric populations and different likely diagnoses, the PSI (Yeh *et al.*, 1984), later evolving into the PRISM score (Pollack *et al.*, 1988). This score, like the APACHE score was validated by its relationship to mortality rates. The PRISM III score is now widely used in a similar way to the APACHE III score (Pollack *et al.*, 1996; Pollack *et al.*, 1997).

Traditional risk factors for mortality in infants receiving intensive care are BW, GA, sex, race and Apgar scores. These parameters alone, however, do not account for differences in outcome between neonatal units. With the motivation to find a grading system that would account for such differences, the SNAP score was developed (Richardson *et al.*, 1993a) (Table 2.6.2) (Richardson *et al.*, 1993b). The elements of the score and the mechanism of scoring were based on those of the APACHE and PSI scores. The physiological parameters were adjusted so that they were appropriate for the newborn. The score was validated against mortality, nursing intensity, length of hospital stay and physician estimate of illness severity. SNAP scores have also been found to correlate with the risk of intraventricular haemorrhage (GrayJE *et al.*, 1992).

There is no 'diagnosis' element within the SNAP score. While diagnostic classifications have been shown to be important in APACHE III and PRISM III scores, it has been argued by Richardson (Richardson *et al.*, 1993b) that the 'diagnostic homogeneity of the very low birth weight population' make it unlikely that diagnosis will play an important role in a scoring system.

The SNAP-II score was developed as a shorter, 6 element, easier to use version of the SNAP score that had as good a correlation with mortality as the original SNAP score. It represents illness severity. Richardson points out that the SNAP score remains a better tool than SNAP-II for studies including mild and moderately ill infants (Richardson *et al.*, 2001). This is because the SNAP-II score retains only severely deranged physiology for scoring, thus giving the strong association with mortality.

The SNAPPE-II score is the SNAP-II score plus weighted scores for birth weight, Apgar score and being small for gestational age. Richardson states that neither the SNAP-II nor SNAPPE-II are designed for sequential use but are admission scores.

The 6 element CRIB score (Table 2.6.3) was designed to be carried out in the first 12 hours after birth on infants with birth weight <1500gr or born <31 weeks gestation and related to mortality (The International Neonatal Network, 1993). It combines 3 physiological parameters with birth weight, gestational age and presence of congenital malformations. Until recently it was only used as the SNAP score had been ie primarily as a tool in ITU performance assessment. A recent study however has shown that the CRIB score is suitable for extended application as an illness severity score rather like the APACHE and PRISM scores. The CRIB score was examined for reliability and validity beyond the first 12 hours, and responsiveness over 7 days (Fowlie *et al.*, 1998). The authors examined the score and 'changes in score' over 7 days for relationships with mortality, prolonged O<sub>2</sub> supplementation and disability at 2 years. CRIB scores taken throughout the first week correlated with all three outcomes. The study showed that a change in CRIB score is associated with mortality risk independent of CRIB score of the first 12 hours. It should, however, be noted that the CRIB score is heavily weighted for GA and BW which remain constant when the score is used longitudinally over time.

Various neonatal illness severity scores and their relationship to mortality have been compared. The variables in the best model to predict mortality were birth weight, birth weight squared, low 5-minute Apgar score, and the SNAP score. The SNAP score was more accurate than the other scores, including CRIB and SNAP-PE-II in predicting mortality (Pollack *et al.*, 2000). However, other researchers have found CRIB scores to be more closely related to mortality than SNAP scores (Rautonen *et al.*, 1994).

Year	Ref	Score	Aim/ use	Time	What measured?	Items	Scoring system	Pop	Numbers	Findings
1993	Richardson (Richards on <i>et al.</i> , 1993b)	SNAP (Score for neonatal acute physiologic y)	Comparison of outcomes between units. Risk assessment independent of diagnosis or therapy	1 <sup>st</sup> 24 hours after admission	Worst physiological derangements in each organ system. Points for high and low values. Total 0-42. Mean 8.7.	26	0=normal 1=needs monitoring 3=alter therapy 5=acute life threatening	All neonates admitted to ITU (excludes infants out of ITU by 24 hours +>48 hrs old)	1643 admission s114 deaths, three centers, prospective, 11 months	SNAP separated into mortality risk groups and had little correlation with birth weight. SNAP correlated with nursing intensity, length of stay, physician estimate of illness severity
2000	Richardson (Richards on <i>et al.</i> , 2001)	SNAP-II	Illness severity. Simplify SNAP. Update SNAP. Increase reliability. Properly weighted to reflect mortality risk.	1 <sup>st</sup> 12 hours after admission	Worst physiological derangements in each organ system. Points for high and low	6	Assigned 5-28 points per parameter	Neonates (excludes infants out of ITU by 24 hours +>48 hrs old)	Validated on 10,819 infants 30 sites	
2000	Richardson (Richards on <i>et al.</i> , 2001)	SNAPPE-II (Perinatal Extension)	Mortality risk	1 <sup>st</sup> 12 hours after admission	Worst physiological derangements in each organ system. Points for high and low +bw, apgar, sga	9	Assigned 5-28 points pre parameter	Neonates (excludes infants out of ITU by 24 hours +>48 hrs old)	Validated on 14,610 infants 30 sites	

Year	Ref	Score	Aim/ use	Time	What measured?	Items	Scoring system	Pop	Numbers	Findings
1993 + 1995	Working groups (The International Neonatal Network, 1993)	CRIB (Clinical Risk Index for Babies)	Develop a tool to compare mortality and morbidity rates and monitor ITU performance and compare populations	1 <sup>st</sup> 12 hours after	Bw, ga, congenital abnormalities +3 physiological variables	6	Various weighted scores	Infants born <1500gr or <31 weeks ga	812	Quick, valid robust tool to predict mortality and major outcome. Non-tertiary hospitals performed worse than tertiary centers.
1992	Gray (Gray <i>et al.</i> , 1992)	NTISS Neonatal Therapeutic Intervention Scoring System	Measure of therapeutic support required. Adapted from TISS. Illness severity score.	Admission day	Therapeutic interventions, not physiology	52	Weighted 0-4	Ventilated Infants	1643	Score correlated with NICU stay, physician estimates of severity of illness, and mortality.
1981	Knaus (Knaus <i>et al.</i> , 1981)	APACHE (Acute physiology and chronic health evaluation)	Comparison of outcomes between units. Tool to be able to control for severity of illness in comparisons of groups of patients.	1 <sup>st</sup> 32 hours after admission	Pre-existing health and acute physiological derangement.	34	Weighted 0-4	Adults in ITU. Not MI or burns.	805 admissions	Significant relationship with therapy required and mortality



Year	Ref	Score	Aim/ use	Time	What measured?	Items	Scoring system	Pop	Numbers	Findings
1985	Knaus <i>et al.</i> , 1985)	APACHE (II)	Comparison of outcomes between units. Assess therapy in trials, evaluate resource use, compare performance.	1 <sup>st</sup> 24 hours after admission	Pre-existing health and acute physiological derangement. 0-71	12	Weighted 0-4	Adults in ITU	5815 patients	Score closely correlated with mortality.
1991	Knaus <i>et al.</i> , 1991)	APACHE III	Comparison of outcomes between units, and sequential measures.			19			17 440	Increase in Apache score increased mortality risk. Daily scoring gave updates on risk estimates.
1984/1987	Yeh (Lee <i>et al.</i> , 1989, Yeh <i>et al.</i> , 1984)	PSI Physiologic Stability Index	Comparison of outcomes between units	1 <sup>st</sup> 24 hours after admission	Most abnormal measurement used for scoring	34	0=normal 1=concern 3=change therapy 5=life threatening	Children in ITU	423	

Year	Ref	Score	Aim/ use	Time	What measured?	Items	Scoring system	Pop	Numbers	Findings
1988	Pollack (Pollack <i>et al.</i> , 1988)	PRISM Paediatric risk of mortality score	Comparison of outcomes between units, assessment of therapies, organising study groups etc. Aim was to reduce amount of data required for PSI	1 <sup>st</sup> 24 hours after admission	Most abnormal measurement used for scoring	14	0=normal. 1=concern. 3=change therapy. 5=lifethreatening	Children in ITU	1415	
1996	Pollack (Pollack <i>et al.</i> , 1996)	PRISM III	Comparison of outcomes between units	1 <sup>st</sup> 12 hours after admission	Most abnormal measurement used for scoring	17	0=normal 1=concern 3=change therapy 5=life threat	Children in ITU	11165	

Table 2-5 Table of literature on illness severity scores

## 2.6.2 SNAP score sheet

				1pt	3pt	5pt	12	pre	act
PH $\leq 7.3$				7.2-7.3	7.10-7.19	<7.10			
pCO <sub>2</sub> $\geq 50$				50-65	66-90	>90			
pO <sub>2</sub> <65				50-65	30-50	<30			
FiO <sub>2</sub>							•	•	•
pO <sub>2</sub> /FiO <sub>2</sub> <3.5				2.5-3.5	0.3-2.49	<0.3			
Max FiO <sub>2</sub> >40				0.41-0.6(1)	0.61-0.9(3)	0.91-1.0(5)	•	•	•
Min FiO <sub>2</sub> >40				0.41-0.6(2)	0.61-0.9(3)	0.91-1.0(4)	•	•	•
Bicarb hi >33				>33					
Bicarb lo <15				11-15	<10				
Min BE <-7.0				>-7.0	-7.01to-10.	-10.1 to-15.	•	•	•
MAP							•	•	•
FiO <sub>2</sub>							•	•	•
pO <sub>2</sub>							•	•	•
O <sub>2</sub> index >7				7-20	21-40	>40			
RR>60				60-100	>100				
BM hi>8.3				8.3-12.9	>12.9				
BM lo<2.2				1.7-2.2	<1.7				
BP max>66				66-80	81-100	>100			
BP min<35				30-35	20-29	<20			
HR max>180				180-200	201-250	>250			
HR min<90				80-90	40-79	<40			
Temp>35.6=n				35-35.6	33.3-35	<33.3			
UOP<1/kg/hr				0.5-0.9	0.1-0.49	<0.1			
Hct high>66				66-70	>70				
Hct low<30				30-35	20-29	<20			
Wcc<5				2.0-5.0	<2.0				
neuts%							•	•	•
acuual n<1				500-999	<500				
Pl<100				30-100	0-29				
SBR >2 kg>256				256.5-342	>342				
SBR<2kg/kg				85.5-171	>171				
Conj SBR>34				>34.2					
Urea n=<14.3				14.3-28.6	>28.6				
Na high>150				150-160	161-180	>180			
Na low<130				120-130	<120				
K high>6.6				6.6-7.5	7.6-9.0	>9.0			
K low<3.0				2.0-2.9	<2.0				
Ca tot hi>3.0				>3.0					

Ca tot low<1.7				1.2-1.7	<1.2				
Cr>106				106-217	217-354	>354			
Seizure				single	multiple				
Apnea				responsive	unresp	complete			
TOTAL									

Table 2-6 SNAP score sheet

### 2.6.3 CRIB score sheet

1st12hr	Score	Actual				
BW			>1350(0)	850-1350(1)	700-850(4)	<700(7)
GA			>24(0)	<24(1)		
Malform.?			none(0)	non-threat(1)	life threat(3)	
Worst BE			≥7(0)	-7.1-10.0(1)	-10.1-15.0(2)	>-15.0(3)
Min O2			≤0.4(0)	0.41-0.6(2)	0.61-0.9(3)	0.91-1.0(4)
Max O2			≤0.4(0)	0.41-0.6(1)	0.61-0.9(3)	0.91-1.0(5)
TOTAL						

Table 2-7 CRIB score sheet

### 2.6.4 SNAP-II and SNAPPE-II components

Mean Blood Pressure

Temperature

PO2/FiO2 ratio

Ph

Multiple seizures

Urine output

### 2.6.5 SNAP Perinatal extension:

BW <750, 750-999

Small for gestational age

Apgar scor at 5 minutes <7

### 2.6.6 Oxygenation Index

$$OI = \frac{MAP(cmH_2O) \times fiO_2(\%)}{PaO_2(mmHg)}$$

## **2.7 Circadian rhythm in cortisol levels in infants.**

### **2.7.1 Studies with data on cortisol circadian rhythm**

Circadian rhythm in cortisol secretion is a characteristic feature of a healthy hypothalamo-pituitary-adrenal (HPA) axis in the child and adult. Sick adults in ITU with high cortisol levels lack circadian rhythm (Reincke *et al.*, 1995). In infants born at term, a cortisol circadian rhythm emerges at 8-12 weeks (literature summary Table 2-8).

There is one longitudinal study of circadian rhythm in cortisol secretion in preterm infants (Antonini *et al.*, 2000). Saliva samples were collected from 9 infants born at 31-34 weeks gestation and continued at home. Circadian rhythm developed at a mean of 9.3 weeks (median 8) and was associated with the development of a circadian rhythm in sleep pattern. There are no similar studies of infants born before 30 weeks gestation.

A cross-sectional study of plasma cortisol levels in 20 infants born at 28-37 weeks gestation made measurements at 0930 and 1530hrs on days 3-4 of life. In that study, afternoon values were significantly lower than morning values for grouped data, but there was a wide variation in cortisol levels, and only 4 out of 20 infants had a 30-50% drop in plasma cortisol in the afternoon (Hindmarsh *et al.*, 1989).

Several studies have suggested that circadian rhythms emerge in preterm infants as a result of environmental cues and are therefore a function of post-natal age rather than maturation dependent post-conceptual age (Antonini *et al.*, 2000; McMillen *et al.*, 1991; Mirmiran and Kok, 1991; Shimada *et al.*, 1993).

### **2.7.2 Potential for circadian rhythm in pre-term infants:**

There is neurological and physiological evidence that preterm infants have the potential to generate circadian rhythms (Rivkees, 1997). The human circadian timing system is believed to arise from the hypothalamus, in particular the paired suprachiasmatic nuclei (SCN). When mature, the SCN exhibit endogenous rhythms based loosely on a 24 hour cycle. Radio-

labelled melatonin has been used to detect the presence of the human SCN at 18 weeks gestation (Reppert *et al.*, 1988). There is also evidence that in some species of mammals, oscillation of the circadian clock begins *in utero* (Reppert, 1992). Transplanted cells from the SCN of animal newborns have been shown to restore circadian rhythm to adults in whom it had been abolished by ablation of the SCN (Hastings, 1998). Maturation of the SCN continues after birth at term, with neuronal cell numbers increasing so that for some cell types, numbers comparable to the adult are not reached until the age of one year (Swaab, 1995). The retinohypothalamic tract has been identified in a human newborn infant at 36 weeks gestation (Glotzbach *et al.*, 1992). This is the neurological pathway by which photic information is relayed from the retina to the hypothalamus in order to keep 24-hour physiological cycles in tune with the environment (Moore, 1983; Morin, 1994). In human fetuses, day-night rhythms can be detected for heart rate, respiratory rate, and adrenosteroidogenesis (Rivkees and Reppert, 1992; Seron-Ferre *et al.*, 1993; Seron-Ferre *et al.*, 2001). Because these rhythms are not detected after birth, however, it has been presumed that they are maternally derived. It may be, though, that postnatal environmental and physiological stimuli manifest as stress, overwhelming and distorting for a while a true endogenous rhythm present in the fetus.

### 2.7.3 Relevance of circadian rhythm in preterm infants:

It has been suggested that preterm infants who are exposed to day-night cycles in the nursery are less ill and grow faster than those without such a light cycle (Mann *et al.*, 1986; Miller *et al.*, 1995). It is conceivable that providing a 'night' enables infants to cope with the 'stress' of daytime stimuli, and that reduction in stress enhances physical and emotional well-being. Thus in this population, circadian rhythm may be an important aspect of health and well-being. Glotzbach *et al.* proposed that an understanding of all aspects of the circadian system may facilitate evaluation of factors that improve therapeutic responses, recovery and outcome of preterm infants (Glotzbach *et al.*, 1995).

Knowledge of the presence and development of circadian rhythm in cortisol secretion in preterm infants may shed light on the degree of organisation and maturity of cortisol secretion and the nature of its central control. It would also be useful for the interpretation of cortisol levels in the newborn period.

First Author	Year	Type of pub <sup>n</sup>	Sample fluid	Type of study	Population	Number of subjects	Age at time of sampling	Values nmol L <sup>-1</sup>	Frequency sampling	of	Circadian rhythm findings
Zurbrugg (Zurbrugg, 1976)	1976	Book Chapt	Blood	Cross section	Term +	6 5 11	3-9 days old 1-6 months 1.5-11.5 years		4 hourly		Appears in 2 <sup>nd</sup> year 12 hourly in neonates
Price (Price <i>et al.</i> , 1983)	1983	Paper	Saliva (+citric acid)	Longitudinal	Term	8	1,4,8,12,16,20, 24 weeks	daily mean 8.6-20 depending on age	0600-0800 1100-1300 1500-1800 2200-2400		Appears by 3 months
Hindmarsh (Hindmarsh <i>et al.</i> , 1989)	1989	Paper	Plasma	Cross section	28-37 weeks	10 stressed 10 un-stressed	about 3 days old	448 mean 294 mean	0930 1530		Present during first few days
Azcarate (Azcarate AB, 2001)	1997	Abstract	Saliva (+citric acid)	Cross-section	Well term Well prem Sick prem	4 7 7	Day3	5-54 depending on group	3 hourly for 24 hours		No circadian rhythm found
Klug (Klug <i>et al.</i> , 1997)	1997	Abstract	Saliva (on cotton swab)	Cross-section	Term Healthy	119	Day1	3.3-116.6 mean 26.3	0800 1300 1800		Overall no circadian rhythm but striking in a small subgroup



First Author	Year	Type of pub <sup>n</sup>	Sample fluid	Type of study	Population	Number of subjects	Age at time of sampling	Values nmol L <sup>-1</sup>	Frequency of sampling	Circadian rhythm findings
Antonini (Antonini <i>et al.</i> , 2000)	2000	Paper	Saliva	Longitudinal	31-34 weeks	9	2,4,8,12,16, 24 weeks	none given	0830 2100	Appears at median of 8 weeks and mean of 9.3 weeks
Santiago (Santiago <i>et al.</i> , 1996)	1996	Paper	Saliva (+citric acid)	Longitudinal	Term	9	2,4,8,12,16, 24 weeks	<1.7-28nmol/l	0800-0900 1700-1800 2100-2200	Appears at mean 8 weeks One at 2 and one at 4 weeks
Spangler (Spangler, 1991)	1991	Paper	Saliva (ascorbic acid and gauze swab)	Cross section	newborn infants	11 14	2-7 days 3,5,7,mo	5.5-13.8 mean depending on time and age	prior to feeds, maximum 4 hourly	Appears at 3 months biphasic in newborns
Kiess (Kiess <i>et al.</i> , 1995)	1995	Paper	Saliva	Cross section	<1 year	10	Various	2-100	0800 1300 1800	Not present before 9 months
Mantagos (Mantagos <i>et al.</i> , 1998)	1998	Paper	Plasma	Cross section	<6mo	40 30	<1 month 1-6 months	mean 99-456 depending on age	1030 2230	Appears between 2 and 3 months

First Author	Year	Type of pub <sup>n</sup>	Sample fluid	Type of study	Population	Number of subjects	Age at time of sampling	Values nmol L <sup>-1</sup>	Frequency of sampling	Circadian rhythm findings
Francis (Francis <i>et al.</i> , 1987)	1987	Paper	Saliva citric	Cross section	Term	8	2 days	2.5-57.4	2hrly samples	No circadian rhythm found Biphasic 12 hourly pattern
Vermes (Vermes <i>et al.</i> , 1980)	1980	Paper	Plasma	Longitudinal	Term	5 in each age group, 45 total	1,2,3 days 1,2,4 weeks 2,3,6 months 1,3 years	mean 406 at 6 months and 783 at 1 day	4 hourly	Appears at 3 months none at 1,2,3 days biphasic at 2 weeks
Onishi (Onishi <i>et al.</i> , 1983)	1983	Paper	Plasma	Cross section	1month-15 years hospitalized	14 10 14 15 11	1mo-5mo 6mo-12mo 1-6 years 6-12 years 12-15 years	39.2 at 1mo-5mo 32.3 at 6mo-12mo	0600 1200 1800 2400	Appears at about 6 months
Economou (Economou <i>et al.</i> , 1993)	1993	Paper	Plasma	Longitudinal: grouped data	Term well Term sick Pre- well Pre- sick	60	Day 1,3,5,10,15,30	142 Twell 268 Tsick 165 Pwell 360 Psick day3 means	0800 1400 2000 0200	Sick and preterm infants had higher levels in the evening

Table 2-8 Summary of literature on cortisol circadian rhythm in infants

### **3 METHODOLOGY**

#### **Laboratory**

Salivary cortisol assay

Plasma cortisol assay

High Performance Liquid Chromatography

Mass Spectrometry

#### **Other**

Statistics

Ethical approval

Informed Parental Consent

Setting

#### **3.1 Use of different assays for plasma and saliva.**

Two different radioimmunoassays were used to measure cortisol in the course of this project; one for saliva, and one for plasma. It would have been ideal to use the same antibody for the correlation of plasma and salivary cortisol, but for practical reasons, this was not done. The reason for this was that it is generally expected that salivary cortisol is present in low concentrations. The salivary cortisol assay that was used was developed specifically to be accurate at these low concentrations. The encapsulated antibody that was key to this feature, was highly specific, but was unfortunately of limited supply and not available for use in the plasma assay, in its 'un-encapsulated' form. The plasma assay was developed and validated for the small volumes of plasma encountered in neonatal research.

#### **3.2 Salivary cortisol assay**

The assay used for the measurement of cortisol in saliva was an in-house radioimmunoassay. It was performed by the staff of the department of Child Life and Health, University of Edinburgh, with the candidate observing. The assay was originally developed by Dr Mike

Wallace, who is based at the Department of Biochemistry, Glasgow Royal Infirmary. Dr Wallace kindly manufactured and donated the encapsulated antibody required for the assay. The assay was carried out without prior extraction.

### 3.2.1 Principle of the function of the 'encapsulated' antibody:

Separation of antibody-bound, labelled cortisol and non-antibody-bound, labelled cortisol was carried out by centrifugation without the need for a second antibody or other separation technique (eg Charcoal). Antibody was encapsulated in a semipermeable nylon membrane containing magnetic dextran (Wallace AM Wood DA, 1984). During centrifugation, the capsules aggregate and accumulate in a 'pellet' at the bottom of the tube.

### 3.2.2 Reagents

**Antiserum:** The antibody was raised in sheep injected with the immunogen cortisol 3 – carboxymethyloxime ovalbumen and was purchased from Guildhay Ltd.

**<sup>125</sup>I-Radioligand:** The radioligand used in the assay was cortisol-3-(O-carboxymethyl) oximo-(2-[I<sup>125</sup>] iodohistamine) methanol:water (9:1) and was purchased from Amersham Life Sciences.

**Cortisol standards:** Cortisol standards were prepared in assay buffer from hydrocortisone purchased from Sigma. Standards were prepared to cover the range 0-32 nmol L<sup>-1</sup> from an initial ethanolic solution of hydrocortisone (0.5mM). The eight point standard curve included the following concentrations: 32, 16, 8, 4, 2, 1, 0.25 and 0 nmol L<sup>-1</sup>.

**Buffer,** The buffer used throughout the assay was phosphate buffered saline with 0.5% sodium azide and 1% gelatine. Phosphate buffered saline (0.05M) pH 7.4 was prepared from concentrated stocks and combined according to Sorensen's recipe to obtain the correct pH. Wash buffer was 0.9% sodium chloride (saline) containing 0.1% gelatine. The wash buffer was prepared fresh for each assay. Sodium citrate, sodium dihydrogen orthophosphate,

disodium hydrogen orthophosphate, sodium chloride and gelatine were purchased from BDH.

### 3.2.3 Freeze-thawing

Four cycles of freeze-thaw for each sample were performed in order to reduce salivary viscosity.

7.25 % difference was apparent after the freeze thaw cycles. This was based on results from 10 samples, each separated into two; one that was freeze-thawed 4 times, and one that was not. This difference is equivalent to the intra-assay coefficient of variation (7.4%).

### 3.2.4 Assay procedure:

- 1) Polystyrene 'ependorph' tubes and precise automatic pipettes were used. All standards and samples were assayed in duplicate.
- 2) The standards and quality controls were prepared in bulk beforehand and stored at -20°C. For each assay, samples, quality controls and standards were thawed and the assay was set up in Starstedt tubes (3ml, 10x75mm).
- 3) Stock antibody was diluted (1:200) with salivary assay buffer to give the optimum antibody titre for the assay. 200 µl of the antibody solution was added to the tubes containing the standards, quality controls and samples.
- 4) Label was freshly prepared for each assay; 10,000 cpm in 100 µl. It was added to all tubes including the 'totals' and non-specific binding tubes that contained no antibody.
- 5) The assay tubes were mixed by vortex and allowed to incubate at room temperature for 90 minutes.
- 6) All assay tubes except the 'totals' were then washed with a wash buffer (saline + 0.1% gelatine) three times. The antibody formed a pellet when centrifuged (3000G, 5 minutes at 4°C) between each wash.

7) After the third wash, the pellet was counted on the Packard Cobra autogamma counter.

The standard curve was plotted and the unknown sample concentrations were calculated from the standard curve.

### 3.2.5 Assay performance

**Sensitivity:** The lower limit of detection for the assay was  $0.25 \text{ nmol L}^{-1}$

**Precision:** The intra-assay co-efficient of variation was 7.4% (Calculated from 10 x single sample in one assay.)

The inter-assay coefficient of variation was 12.78 %, 14.07% and 7.37 % at  $2 \text{ nmol L}^{-1}$ ,  $6 \text{ nmol L}^{-1}$  and  $16 \text{ nmol L}^{-1}$ . This figure was obtained from the low, medium and high quality control (QC) included on each assay run.

### 3.2.6 Specificity of salivary cortisol RIA antibody

Steroid	% Specificity
Cortisol	100
Prednisilone	31
Corticosterone	18.6
Fludrocortisone	2
11-deoxycortisol	0.85
17- $\alpha$ -hydroxyprogesterone	0.3
Prednisone	<0.1
Cortisone	<0.1
Dexamethasone	<0.1
11-deoxycorticosterone	<0.1
Pregnenolone	<0.1
Spironolocatone	<0.1
Progesterone	<0.1
Testosterone	<0.1
17 $\beta$ -Oestradiol	<0.1
Tetrahydrocortisol	<0.1
Cholesterol	<0.1

Table 3-1 Cross reactivities of antibody used in salivary cortisol assay

3.2.7 Standard curve for Salivary cortisol RIA

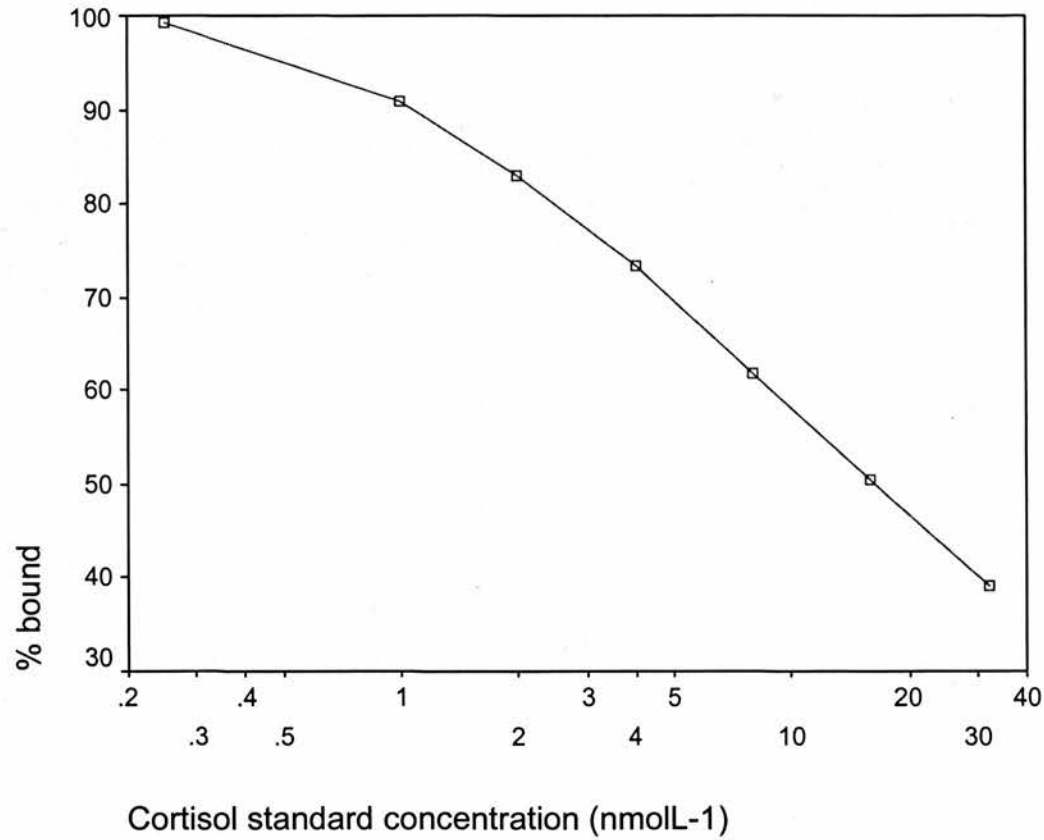


Figure 3-1 Standard curve for Salivary cortisol RIA

### 3.3 Plasma cortisol assay

Plasma cortisol was measured by an in house 'micro' radioimmunoassay, specifically developed for the small volumes encountered in neonatal research. The assay measured total cortisol in 10  $\mu\text{l}$  of plasma (i.e. 5  $\mu\text{l}$  in duplicate). It was a competitive radioimmunoassay with separation of bound and free label by second antibody precipitation. No prior extraction was performed. The pH was kept less than 4 during the assay to ensure that cortisol was detached from cortisol binding globulin, in order to be detected by the assay.

#### 3.3.1 Reagents

**Antisera:** The primary antibody was provided by the Scottish Antibody Production Unit (SAPU), now Diagnostics Scotland. It was raised in sheep immunised with cortisol-3-O (carboxymethyl) oxime-bovine albumin conjugate.

Double antibody precipitation utilised donkey anti-sheep sera and non-immune sheep serum, obtained from SAPU.

**$^{125}\text{I}$ -Radioligand** The radioligand used in the assay was cortisol-3-(O-carboxymethyl) oximo-(2-[ $^{125}\text{I}$ ] iodohistamine) methanol:water (9:1) and was purchased from Amersham Life Sciences.

**Cortisol Standards:** Cortisol standards were prepared in assay buffer from hydrocortisone purchased from Sigma. Standards were prepared to cover the range 0-500  $\text{nmol L}^{-1}$  from an initial ethanolic solution of cortisol (0.1mM). The thirteen point standard curve included the following concentrations: 500, 200, 100, 50, 20, 10, 5, 2, 1, 0.5, 0.2, 0.1 and 0  $\text{nmol L}^{-1}$

**Buffer:** The buffer used throughout the assay was citrate phosphate buffer with 0.1% gelatine, 0.1M, pH 4.00. Sodium citrate, sodium dihydrogen orthophosphate, disodium hydrogen orthophosphate, sodium chloride and gelatine were purchased from BDH.



### 3.3.2 Assay procedure

1. Blood samples were collected into lithium heparin tubes, centrifuged and the plasma pipetted into eppendorf tubes. The samples were stored at -20°C until analysis.
2. Polystyrene 'ependorph' tubes and precise automatic pipettes were used. All standards and samples were assayed in duplicate.
3. The standards and quality controls were prepared in bulk beforehand and stored at -20°C and were diluted (1:60) prior to use. For each assay, they and the samples were thawed and added to Starstedt tubes (3ml, 10x75mm).
4. The primary antibody was diluted 1:4000 in assay buffer from a 1:100 stock antibody to give a final dilution of 1:32 000 in the assay. Antibody (50µl) was added to all tubes except totals, and NSBs.
5. Label was freshly prepared for each assay; 10,000 cpm in 50 µl. The label was added to all tubes including the totals and NSB tubes.
6. The assay tubes were mixed by vortex and allowed to incubate at room temperature for 4 hours.
7. Before the incubation was complete, the second antibody precipitate was prepared. Donkey anti-sheep/goat sera (DAGS) in a final dilution 1:28, non-immune sheep sera (NISS) in a final dilution 1:400 were combined.
8. At the end of the incubation 100µl of this DAGS/NISS was added to all tubes except the totals. The assay tubes were vortexed and incubated at 4°C for 18 hours.
9. The bound fraction was obtained after centrifugation at 4°C for 45 minutes at 3000G.
10. The supernatant was aspirated.

11. The precipitate was counted on the Packard Cobra autogamma counter. The standard curve was plotted and the unknown sample concentrations were calculated from the standard curve.

### 3.3.3 Assay performance

**Sensitivity:** The minimum detection of the assay was 29 nmol L<sup>-1</sup>

#### Precision

The intra-assay coefficient of variation was 8.42 % this figure was obtained from a sample run ten times in one assay.

The inter-assay coefficient of variation for the assay was 11.2 %, 12.9% and 11.2 % at 77, 280 and 765 nmol L<sup>-1</sup> This figure was obtained from the low, medium and high quality control (QC) included on each assay run.

### 3.3.4 Standard curve for Plasma cortisol assay

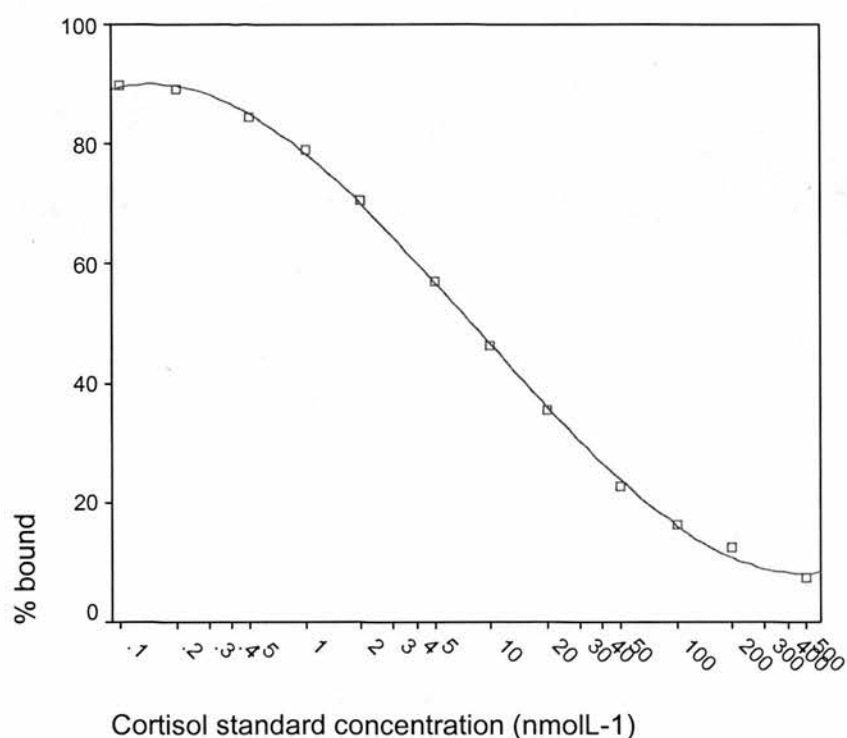


Figure 3-2 Standard curve for plasma cortisol assay

### 3.3.5 Specificity of plasma cortisol RIA

Steroid	Cross reactivity
Cortisol	100
Prednisolone	48
21-deoxycortisol	34
Fludrocortisone	11
17 $\alpha$ -hydroxyprogesterone	5
11-deoxycortisol	3
6 $\beta$ -hydroxycortisol	1
$\alpha$ -cortolone	<0.61
Dexamethasone	0.5
20 $\beta$ -dihydrocortisol	<0.49
20 $\alpha$ -dihydrocortisol	<0.49
Tetrahydrocortisol	0.41
$\alpha$ -cortol	0.34
21-deoxycortisone	0.3
Corticosterone	0.18
$\beta$ -cortol	<0.17
Prednisone	0.16
Cortisol-21-glucuronide	<0.15
20 $\beta$ -dihydrocortisone	0.14
20 $\alpha$ -dihydrocortisone	<0.09
Spironolactone	<0.09
Canrenone	<0.09
Progesterone	<0.09
Testosterone	<0.09
Androstenedione	<0.09
Pregnenolone	<0.09
Tetrahydrocortisone	<0.09
Aldosterone	<0.09
Dehydroepiandrosterone sulphate	<0.09
Cortisone	0.07
11-deoxycorticosterone	0.03

Table 3-2 Cross reactivities of antibody used in plasma cortisol assay

### 3.4 HPLC

#### 3.4.1 Reagents and Equipment

The HPLC equipment comprised a Waters Quarternary pump, Waters 600E System controller, linked to a computer running Millenium v2.0 software and a Waters 486 tunable Absorbance detector. The column was a C-18  $\mu$ bondapak column and the mobile phase was methanol:water (1:1). Fraction collection was carried out manually, and the pumps were calibrated to deliver  $1.0 \text{ ml min}^{-1}$ . Ethyl acetate, methanol and sodium hydroxide were obtained from BDH.

#### 3.4.2 Sample Extraction

A small volume of saliva was diluted in distilled water so that the final working volume was  $500 \mu\text{l}$ . The diluted sample was added to glass conical extraction tubes (Quickfit). The steroids were extracted into 2 mls of ethyl acetate. Extraction was achieved by vigorous inversion. The aqueous layer was separated by centrifugation at 2000 G for 10 minutes. The extract was washed firstly with  $500 \mu\text{l}$  0.1M sodium hydroxide and secondly with  $500 \mu\text{l}$  distilled water. A known volume ( $750 \mu\text{l}$ ) of the ethyl acetate extract was transferred to two glass tubes and evaporated to dryness under nitrogen. The extract was then re-suspended in  $100 \mu\text{l}$  of the mobile phase (methanol:water (1:1)).

#### 3.4.3 HPLC purification

The HPLC column was prepared by equilibrating with the methanol:water (1:1) running through the system at least an hour before the first run. The first run was always to check the separation of cortisol and cortisone. A cortisol:cortisone mixture was injected on to the column. The absorbance was monitored and the retention time for both cortisol and cortisone was noted. Forty 'one minute' fractions were collected for each sample injection.

The sample was injected on to the column (80  $\mu$ l) and the timer started, 'one minute' fractions were collected. At the end of collection, another injection of cortisol:cortisone was prepared and loaded on to the column to verify that there was no drift in the retention times of both cortisol and cortisone.

The fractions were then back extracted into ethyl acetate. The methanol was evaporated off under a stream of nitrogen and the remaining aqueous volume (50%) was extracted with into 5 mls of ethyl acetate. A known volume of the ethyl acetate extract (1ml) was then evaporated to dryness under a stream of nitrogen and resuspended in assay buffer.

The fractions were then assayed for cortisol using the plasma cortisol assay.

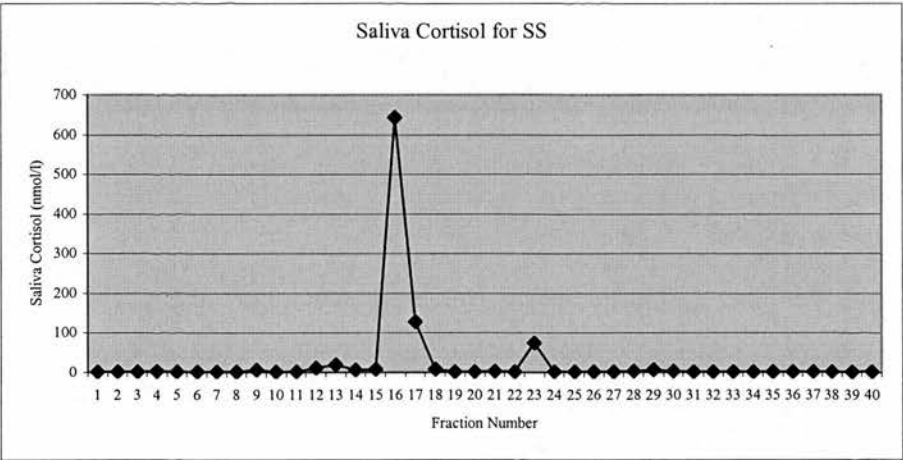


Figure 3-3 Chromatogram from saliva sample showing peak of cortisol

### 3.5 Mass Spectrometry

This is a highly accurate means of identifying the nature and abundance of a substance at a molecular level. Mass spectrometry was kindly carried out by associates at the Western General Hospital, Edinburgh.

#### 3.5.1 Principle

The sample is first injected into a gas chromatograph column. This then directs the vaporised sample to the ionisation chamber. Molecules are converted by the impact of the high-energy electrons into positive ions. An excess of energy remains in the molecular ion causing rupture of one or more bonds. Depending on the amount of energy, some molecular ions may dissociate into fragment ions. A 'repeller' voltage followed by accelerating potential are then applied to the ions so that they may be injected next into the mass analyser. This separates ions according to their mass: charge ratio. This is done by a quadrupole, consisting of 4 parallel conducting rods. Depending on the degree of rod separation and amplitude and frequency of the fields, the ions will follow a predictable trajectory between the rods prior to exiting the quadrupole mass filter. The ions then hit the detector. The detector consists of resolving slit, plate collector and amplifier (electron multiplier).

The equipment is set to scan for set mass ranges. The mass spectrum of a compound contains the masses of the ion fragments and the relative abundance of these ions compared to the parent ion. These occur in the same relative abundance for each ion. A 'Total ion Chromatogram' is reconstructed from the data. It looks similar to a gas chromatogram, and allows for identification of mass spectra for equivalent GS peaks.

## 3.6 Other

### 3.6.1 Statistics

Advice for all statistical analysis was obtained from a medical statistician. Data was stored and all analysis carried out using the statistics software package, 'SPSS' version 10.0. All data handling, analysis and presentation were carried out by the candidate. This was with the exception of the 'mixed model' analysis and multiple subject characteristic correlations (chapter 7), which were carried out by the previously mentioned statistician.

### 3.6.2 Ethical approval

Ethical approval for all parts of the study was obtained from the Lothian Region Ethics Committee.

### 3.6.3 Informed parental consent

Consent for all subjects taking part in the study was obtained from at least one of his or her parents following provision of a verbal explanation and a written information sheet. All consent was written, and witnessed by a member of the neonatal unit staff.

### 3.6.4 Setting

All subjects were in-patients at the Simpson Memorial Maternity Pavilion, Royal Infirmary of Edinburgh. All except the term controls described in chapter 7 were being cared for on the neonatal unit. The neonatal unit is a tertiary referral centre with 12 intensive care cots, 8 'second level' nursery cots, and 24 'special care' cots. There are 6 500 deliveries *per annum* in the hospital, and 477 infants cared for each year in the neonatal unit.

### 3.6.5 Gestational age

GA was based on first trimester ultrasound scan data from the maternal obstetric notes

## 4 Effect of saliva collection method on cortisol radioimmunoassay and subsequent selection of collection method for use in new-born infants.

### 4.1 OVERVIEW

**Background:** Various collection methods can make saliva collection from infants technically easy but may affect cortisol radioimmunoassay performance.

**Objective:** To assess 3 potential saliva collection methods for the neonatal population.

**Method:** Cortisol was measured in saliva collected from 16 adults using;  
cotton swabs, polyester swabs and citric acid

and compared to saliva dribbled directly into a pot (plain saliva).

In infants, aspiration of saliva was piloted using citric acid as a sialogogue.

**Results:** Cortisol in saliva collected using cotton and polyester swabs was significantly higher than cortisol measured in plain saliva ( $p < 0.01$ ). Cortisol in saliva collected using citric acid was not significantly different from plain saliva ( $p = 0.997$ ). Infants appeared to find the use of citric acid distressing.

#### **Conclusions:**

1. The use of cotton or polyester swabs for collection of saliva from infants for cortisol measurement using the radioimmunoassay in this study would be inappropriate.
2. Citric acid stimulation was not adopted in the standard method because of associated infants' distress.
3. The method selected as most appropriate was gentle suction of 'plain' saliva from an infant's mouth using wall mounted electrical suction. The saliva accumulated in the suction trap and was drained into a sample tube.



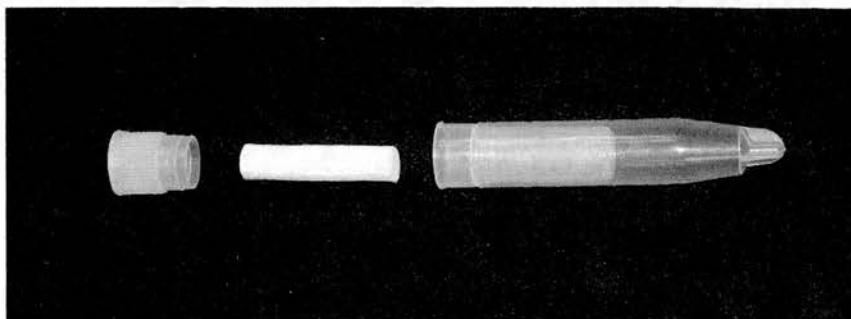
## 4.2 BACKGROUND

As described in chapter 2.5, the results from different studies of plasma cortisol in preterm and sick infants are often conflicting, and the number of infants studied is relatively small. The requirement for repeated blood sampling has been a major factor influencing the quantity, quality and conclusiveness of cortisol data from these infants. Salivary cortisol measurement is now technically accessible to non-specialist laboratories (Al Ansari *et al.*, 1982) and is widely accepted as a reliable indicator of adrenocortical activity in children and adults (Bober *et al.*, 1988; Laudat *et al.*, 1988; Riad-Fahmy *et al.*, 1982; Vining *et al.*, 1983b). There are many studies examining salivary cortisol in term and well infants (Klug *et al.*, 1997; Francis *et al.*, 1987; Spangler and Scheubeck, 1993; Davis and Emory, 1995a; Lewis and Thomas, 1990; Gunnar *et al.*, 1995; Kurihara *et al.*, 1996) but relatively few with data from sick and preterm infants (Antonini *et al.*, 2000; Bettendorf *et al.*, 1998; Azcarate *et al.*, 1997; Calixto *et al.*, 2000; Vaughn PR, 1996a). Salivary cortisol has the potential to expand the knowledge of adrenocortical activity in sick and preterm infants. Repeated, non-invasive, low volume saliva samples would allow more infants to be studied more frequently over longer periods of time than opportunistic or infrequent blood sampling regimes.

In the neonatal population, direct aspiration of saliva can be difficult. Infants in the first few days of life are relatively water depleted, and so their mouths may be too dry to allow suction of collections of saliva directly. Direct suction also requires specialist equipment. Citric acid is widely used as a sialogogue in studies of infant salivary cortisol (Chang *et al.*, 1995; Davis and Emory, 1995a; Francis *et al.*, 1987; Grauer, 1989; Gunnar *et al.*, 1995; Kurihara *et al.*, 1996; Price *et al.*, 1983; Santiago *et al.*, 1996).

Cotton swabs may be placed in an infant's mouth to absorb saliva. These may then be squeezed in a syringe by the syringe plunger so that saliva drips into a collection pot, or centrifuged into the bottom of a collection chamber in a purpose made device, eg the 'Salivette' device (Sarstedt, Numbrucht, FRG).

Figure 4-1 Photograph of a 'Salivette' with cotton swab insert



Polyester swabs were introduced for use in the same way as cotton swabs because the constituents of cotton swabs were found to affect the results in some salivary chemical measurements. It was thought that the polyester swabs were likely to have less 'interfering substances' that might affect the measurements (Lenander-Lumikari *et al.*, 1995). An advantage of swab-collected saliva is that the process of extracting the saliva essentially filters it, giving a non-viscous, clear sample that is free of cellular and other oral debris. This is more pleasant and technically easier to handle in the laboratory.

Also, the use of either cotton or polyester swabs avoids the use of suction equipment. This equipment may be in the form of a traditional 'mucous extractor' with the operator sucking by mouth on one end of the device, but the potential for the transmission of infection such as HIV or Hepatitis B makes this unethical and impractical. Electrical suction devices with sterile, flexible, disposable catheters, of the kind routinely used to clear endotracheal tubes and nasal or nasopharyngeal airways, are an alternative. The devices are usually wall-mounted, as at each cot space in the neonatal unit, but may be portable, like those used by ambulatory resuscitation staff. Both are specialised and relatively expensive. The advantage

of electrical suction is that there is a constant, easily controlled negative pressure that can be set at any level. A thumb over the hole at the 'trap' of the suction catheter completes the seal required for suction, and with small movements of the thumb, the operator can obtain instant and subtle changes in suction.

Figure 4-2 Wall-mounted suction device

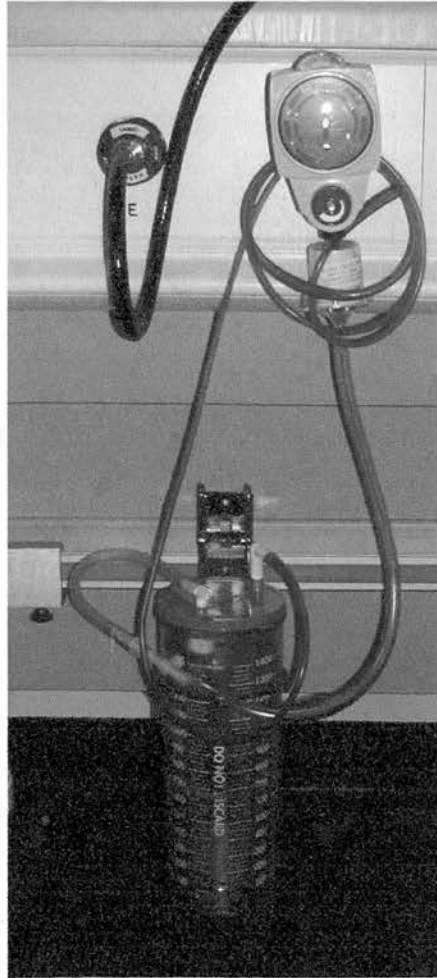
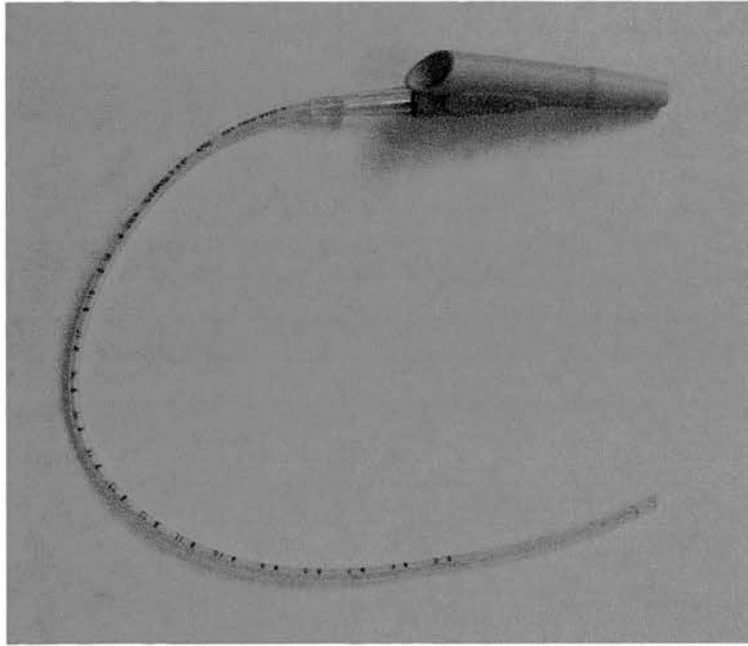


Figure 4-3 Disposable suction catheter



In this study, adults were used for practical and ethical reasons. It takes up to 5 minutes to aspirate sufficient saliva for assay from an infant's mouth and seconds for an adult to spit into a pot. It is difficult to place both a cotton and polyester swab simultaneously in an infant's small mouth. This may be distressing and if so the adrenocortical response to this distress could increase salivary cortisol over the collection time. It is also possible that the infant handling required to collect several consecutive samples may elicit a cortisol response (Davis and Emory, 1995a). Simultaneous, non-stressed samples would thus be unobtainable.

For the radioimmunoassay in this study, 100 $\mu$ l of saliva are required for each assay tube. Therefore 200 $\mu$ l are required for the assay to run in duplicate. Ideally, a minimum of 250 $\mu$ l are collected to allow for viscosity and practical handling of small samples. More is collected if possible to allow further aliquots to be diluted and re-assayed if the initial salivary cortisol measurement is higher than the accurate part of the RIA curve.

### **4.3 OBJECTIVES**

To assess 3 saliva collection methods, in adults, potentially useful in the neonatal population.

With the data produced, to select a collection method appropriate for use in the newborn population of the present study.

### **4.4 METHODS**

#### **4.4.1 Subjects**

16 healthy adults. Volunteers from the neonatal unit staff without endocrine disorders or steroid therapy. Fasted for 1 hour prior to saliva sampling.

10 preterm infants in the neonatal unit. Any infant in the intensive care section of the unit who was not receiving exogenous steroid therapy was recruited for the collection of a single saliva sample.

#### **4.4.2 Saliva collection**

Adult subjects had a cotton swab placed in one cheek, and a polyester swab in the other.

The subject dribbled simultaneously into a 'universal container'. The swabs were removed after one minute, and a few grains of citric acid were placed on the tongue to stimulate the next sample. The citric acid was obtained from the hospital pharmacy department and was prepared to BP standards and purity. The citric acid stimulated sample was dribbled into a second 'universal container'.

#### **4.4.3 Sample handling and storage.**

The swabs were centrifuged at 2000 rpm for 2 minutes within an hour of collection to give clear saliva. All samples were transferred into polythene 'ependorf' tubes and frozen at -20°C until analysis.

4.4.4 Cortisol measurement

Cortisol was measured by in-house direct RIA in duplicate 100 µl aliquots using the method described in chapter 3. All samples were analysed in a single assay run.

4.5 RESULTS:

4.5.1 Salivary cortisol levels

Figure 4-4 Salivary cortisol following different collection methods (nmol L<sup>-1</sup>)

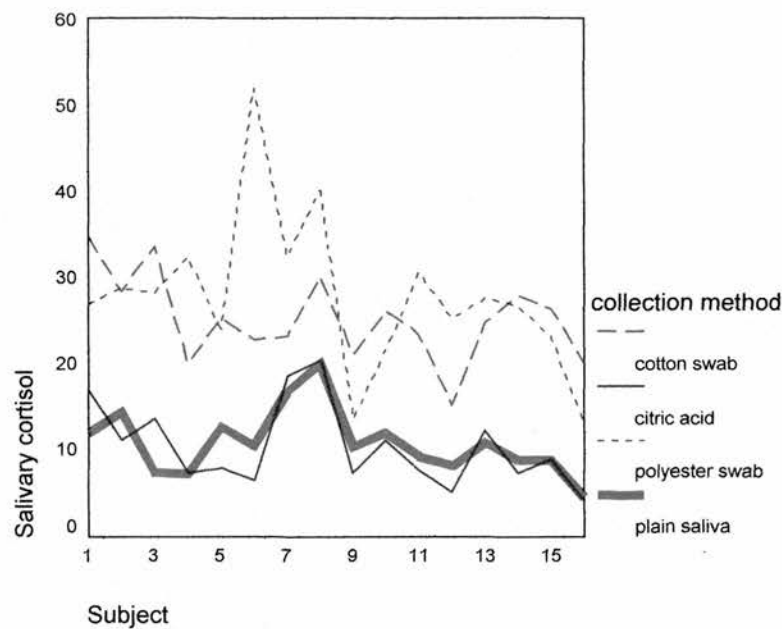


Table 4-1 Results of salivary cortisol radioimmunoassay following different collection methods

n=16	Mean nmol L <sup>-1</sup>	Standard deviation
Plain saliva	10.9	3.8
Citric Acid stimulated	10.4	4.8
Cotton Swab	25.3*	5.5
Polyester swab	27.9*	9.2

ANOVA: \* = significantly higher than plain saliva p<0.01.

#### 4.5.2 Suction of saliva from infants using citric acid as a sialogogue

Most infants screwed up their faces and wriggled when citric acid was placed on the tongue seeming to find the experience distressing. The citric acid increased saliva flow.

### 4.6 DISCUSSION

#### 4.6.1 Effect of swab-collection on Radioimmunoassay

Saliva collected using cotton and polyester swabs resulted in variable and significant over-estimation of cortisol when compared to plain saliva. This was presumed to be due to cross reactivity of swab constituents with the assay antibody. A similar effect was noted with variable over-estimation of testosterone (Walker *et al.*, 1990), and 17OHP (Kruger *et al.*, 1996) by radioimmunoassay after Salivette saliva collection. Constituents of cotton swabs have also been found to affect the measurement of various non-steroid saliva components (Arglebe, 1989). While the effect we found was that of pseudo-activity in the radioimmunoassay, others have found that absorption of the steroid by swab fibers has led to unacceptably low recovery from polyester swabs (Walker *et al.*, 1990).

It is unlikely that the high "swab-collected" cortisol is a genuine stress response. The collection was not perceived to be distressing by the adult volunteers and the 'citric acid' samples did not differ significantly from the plain saliva and were collected *after* the swab samples. The magnitude of the difference between the 'swab' and plain saliva samples and the degree of variation, however, could be interpreted under study conditions as an appreciable stress response in infants (Lewis and Thomas, 1990). The described effect will vary from assay to assay as described by Shirtcliff *et al.* (Shirtcliff *et al.*, 2001). Cotton swab saliva collection has been widely used in infant salivary cortisol studies (Gunnar *et al.*, 1995; Kurihara *et al.*, 1996; Lewis and Thomas, 1990) and may account for some of the conflicting results in the literature. The use of different collection methods and the effect described here also make the comparison of salivary cortisol levels from different publications more

difficult. These results highlight the need for authors of infant salivary cortisol studies to publish careful validation of their collection methods in relation to their radioimmunoassays.

#### 4.6.2 Citric acid and apparent infant distress

Citric acid stimulated samples were not significantly different from the plain saliva. After describing the effect of cotton and polyester swabs on cortisol results, and concluding that swabs were not suitable, collection of saliva from infants using citric acid and direct aspiration using electrical suction was commenced. There was an appreciable effect on infant behaviour. Most infants screwed up their faces and wriggled when citric acid was placed on the tongue seeming to find the experience distressing. Because the use of citric acid in infants had been widely described (Chang *et al.*, 1995; Davis and Emory, 1995a; Francis *et al.*, 1987; Grauer, 1989; Gunnar *et al.*, 1995; Kurihara *et al.*, 1996; Price *et al.*, 1983; Santiago *et al.*, 1996) and the issue of distress had not been identified as a significant factor in those papers, this finding was unexpected. There was therefore no plan for formal definition or assessment of the degree of distress or the recording of the precise number of infants displaying distress. The effect, however, was of a magnitude such that the candidate decided that it was unethical to continue its use after 10 samples were collected from 10 infants. Citric acid is a potent, rapidly-acting sialogogue. It is a main ingredient, along with sugar, in sherbet. The sensation of its presence in the mouth can be intense, with tingling and 'fizzing'. The interpretation of these sensations is subjective. For children with sherbet dips, the association is with fun and other confectionary, and so distress is not usually a feature. Several adults in this study commented that the sensation of citric acid alone was unpleasant.

#### 4.6.3 Description of collection technique selected.

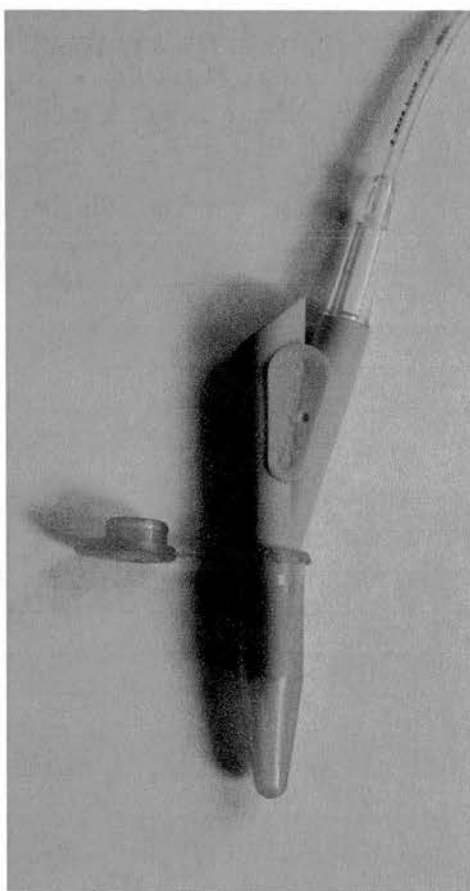
Saliva sampling from infants took from a few seconds to five minutes to perform. Some constituents of milk are known to cross react with cortisol radioimmunoassays (Magnano *et al.*, 1989), so at least 40 minutes was allowed to pass after the last feed to minimise the risk of milk contamination. The negative pressure on the wall-mounted suction device was set to



40 mm Hg. The tip of a size 8 sterile disposable suction catheter was gently introduced to the mouth of the infant, with the operator's thumb off the trap, ie with no suction applied. Often the infant would start to suck on the tube. In this case, the tube was held so that the tip was about 1 –1.5cm from the lips and directed to the roof of the mouth so that it lay between the infant's tongue and palate. With each movement of the infant's tongue on the palate and tube, once the suction was commenced, a small amount of saliva travelled back down the tube towards the suction trap. If the infant did not suck on the tip of the suction catheter, the tip was placed in the dependant parts of the mouth and gently moved around. As relatively low pressures were used, only when there was copious saliva did any leave the trap and travel further back down the collecting system. Because of the ease of control of the suction with the thumb cover on the trap, it was possible to place the tube in position before gently and intermittently at first commencing suction, to minimise any disturbance to the infant. Saliva collection was not considered by parents or nursing staff to be distressing to the infant. Many infants stayed asleep while collection took place. Those who were alert to start with responded with movement +/- vocalisation in the way they would respond to any other handling or stimulation.

Once there appeared to be sufficient saliva in the suction trap, the catheter was removed from the rest of the collecting system, the empty flexible tube cut off, and the trap tipped into and taped to an ependorph tube.

Figure 4-5 Collection trap taped to ependorf tube



To ensure that the maximum volume of saliva was transferred from the trap to the tube, the tube and trap together were then centrifuged for 1 min at 2000rpm. The trap was then removed, the ependorph tube sealed and transferred to the -20°C freezer until analysis.

The advantages of the technique described above are:

1. Avoiding the introduction of error due to interfering substances in cotton and polyester swabs.
2. Avoiding possible distress caused to infants due to citric acid, so making the technique ethical and more acceptable to staff and parents.

The disadvantages of the technique described above are:

1. It takes practice and patience. Non-researchers would require training.
2. Some infants simply will not have sufficient saliva to collect, and so failure to obtain a sample must be accepted as part of any study or clinical programme.
3. Specialist equipment, i.e. electrical suction pump, suction catheters and a centrifuge are required. This makes it less portable, economical and accessible than, for example cotton swab use.

In light of the above, this technique as it stands is most suited to the research setting. Changes in RIA procedure, with thorough assessment of the effects of swab constituents, and/or experimentation with different swab materials might allow a swab technique to be used in this population in the future. Before the technique is used clinically across centers, standardization, simplification and validation of collection method in conjunction with radioimmunoassay should be performed. This should also take into account the practicalities and potential difficulties of reagent supply for extended time periods. The amount of work involved would be appreciable, both in clinical and laboratory terms.

#### **4.7 CONCLUSIONS**

Cotton and polyester swabs may lead to variable overestimation of salivary cortisol, while citric acid does not appear to affect the RIA in this study. Citric acid caused distress to infants. For use in the specialised research setting of a neonatal unit, direct aspiration of saliva by electrical suction without the use of citric acid was selected as the most suitable method of saliva collection.

## 5 Correlation of Salivary and Plasma Cortisol in Sick and Pre-term Infants.

### 5.1 OVERVIEW

**Background** Salivary cortisol is a potentially useful tool for assessing adrenocortical function in sick and pre-term infants.

**Objectives** In this population;

- 1) To establish the range of salivary cortisol levels.
- 2) To correlate salivary and total plasma cortisol.

**Design** Cross-sectional observational study.

**Subjects** 32 infants on the neonatal unit in a tertiary referral center. Median gestational age; 30 weeks, median post-natal age; 0.57 weeks (4 days).

**Measurements** Cortisol was measured by radioimmunoassay in 50 simultaneously gathered saliva-plasma pairs.

### Results

Total plasma cortisol; median 244.2 nmol L<sup>-1</sup>, range 37.3-3802 nmol L<sup>-1</sup>, interquartile range 94.1-418.0 nmol L<sup>-1</sup>

Salivary cortisol; median 43.1 nmol L<sup>-1</sup>, range 2.8-3767 nmol L<sup>-1</sup> interquartile range 14.0-121.3 nmol L<sup>-1</sup>

Total plasma and salivary cortisol were significantly correlated,  $p < 0.01$  ( $r = 0.75$ )

### Conclusions

1. Salivary cortisol levels were wide-ranging and higher than previously reported for any population.
2. Salivary and total plasma cortisol levels were closely and significantly correlated.

## 5.2 BACKGROUND

The levels and dynamics of salivary cortisol in sick and pre-term infants are potentially different from those of term and well infants. There are many publications with salivary cortisol data from term and well infants (Davis and Emory, 1995a; Francis *et al.*, 1987; Gunnar *et al.*, 1995; Kiess *et al.*, 1995; Klug *et al.*, 1997; Klug *et al.*, 2000; Kurihara *et al.*, 1996; Price *et al.*, 1983; Spangler and Scheubeck, 1993) and few with data from pre-term infants (Antonini *et al.*, 2000; Azcarate *et al.*, 1997; Bettendorf *et al.*, 1998; Calixto *et al.*, 2000; Vaughn PR, 1996a). These studies are reviewed in chapter 2d.

## 5.3 METHODS

### 5.3.1 Subjects

A 'convenience sample' was recruited over several days. The aim was to include a cross-section of infants typically resident in a tertiary referral neonatal unit.

### 5.3.2 Inclusion criteriae

Any infant on the neonatal unit who required blood-sampling for clinical reasons.

Parent available for consent.

Researcher available to collect saliva.

### 5.3.3 Exclusion criteriae

Congenital abnormality

Dysmorphism

Exogenous steroid therapy

### 5.3.4 Sample collection and analysis

Saliva was collected immediately prior to blood sampling as described in chapter 4. Blood sampling was by venepuncture or from indwelling arterial catheters.

Cortisol was measured in saliva and plasma as described in chapter 3.

HPLC: 2 samples of saliva from 2 infants with cortisol levels  $>1000 \text{ nmol L}^{-1}$  by radioimmunoassay were purified by high performance liquid chromatography as described in chapter 3.

MASS SPECTROMETRY: The samples analysed by HPLC were then analysed by Mass Spectrometry, also described in chapter 3.

5.3.5 Statistics

Spearman’s rho correlation was used to analyse the relationship between saliva and cortisol because of the skewed distribution of results.

5.4 RESULTS

5.4.1 Subjects

The details of the study infants are summarised in table 5-1. 32 infants were recruited; 50 pairs of saliva and plasma were collected. 25 samples were from infants whose mothers had been treated with a full course of antenatal steroids to prevent respiratory distress syndrome. 18 samples were from infants of mothers who received a partial course of antenatal steroids before giving birth.

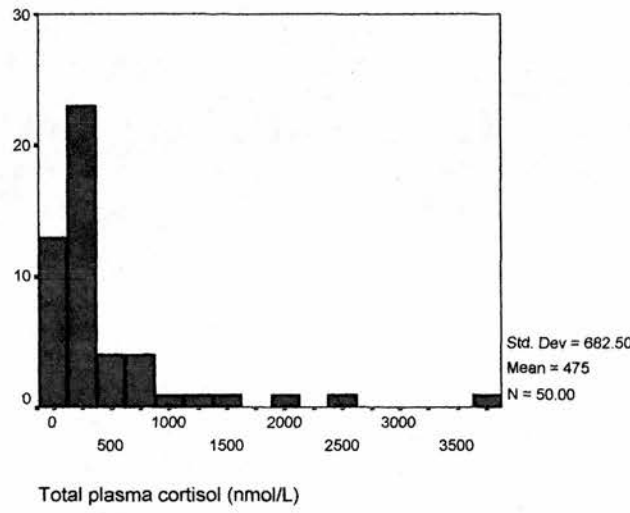
Table 5-1 Characteristics of the 19 male and 13 female infants studied

	Mean	Median	Range
Birth weight (kg)	1.455	1.338	0.595-3.79
GA (weeks)	29.7	30	24-41 weeks
Postnatal age (weeks)	1.74	0.57	0-9

5.4.2 Cortisol

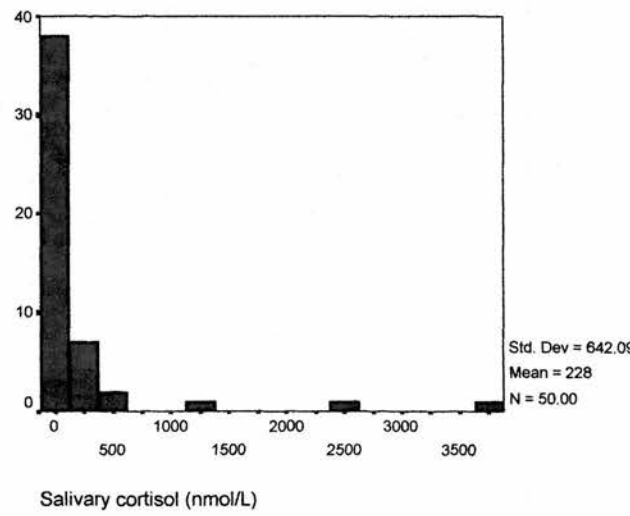
Both total plasma and salivary cortisol had a skewed distribution (figs 5-1 and 5-2).

Figure 5-1 Histogram of total plasma cortisol (nmol L<sup>-1</sup>)



Plasma cortisol: Median 244.2 nmol L<sup>-1</sup>, range 37.3-3802 nmol L<sup>-1</sup>

Figure 5-2 Histogram of salivary cortisol (nmol L<sup>-1</sup>)

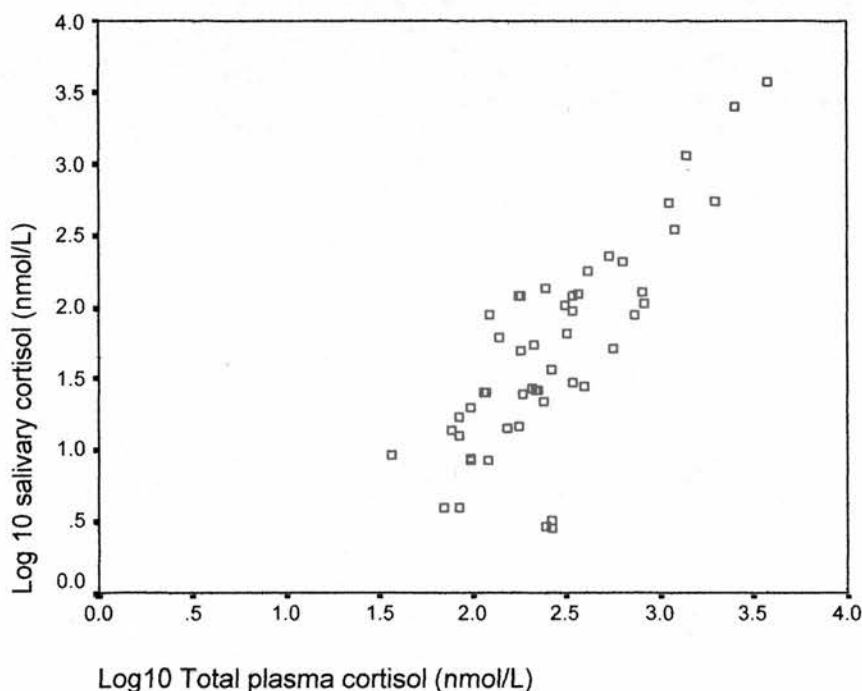


Salivary cortisol: Median 43.1 nmol L<sup>-1</sup>, range 2.8-3767.4 nmol L<sup>-1</sup>

### 5.4.3 Saliva and Plasma pairs

A Scatter Plot of saliva and plasma pairs is shown in fig 5-3. Log10 values are used to separate the cluster of values at the lower end of the scales.

Figure 5-3 Scatterplot of log10 salivary and total plasma cortisol (nmol L<sup>-1</sup>)



Overall Spearman's rho correlation between total plasma and salivary cortisol was significant at  $p=0.01$  level,  $r=0.75$ .

Overall regression equation: salivary cortisol =  $0.87 \times$  plasma cortisol - 186.4

Spearman's rho correlation coefficient for the relationship between plasma and saliva cortisol when total plasma cortisol  $<200$  nmol L<sup>-1</sup> was 0.67 ( $n=20$ ) and when  $>200$  nmol L<sup>-1</sup> was 0.8 ( $n=30$ ) (each significant  $p=0.01$ )



### 5.4.4 HPLC

Values within 10% of the original assay measurement were obtained after purification. Dilutions of the fraction gave predicted values in both salivary and plasma assays, producing straight-line plots of dilution against cortisol, parallel to the plot of dilutions of a commercially obtained cortisol standard (fig 5.4).

Figure 5-4 Plot of serial dilutions of salivary cortisol measured by radioimmunoassay after HPLC purification ( $\text{nmol L}^{-1}$ ). Series 2=standard, series 3=saliva.

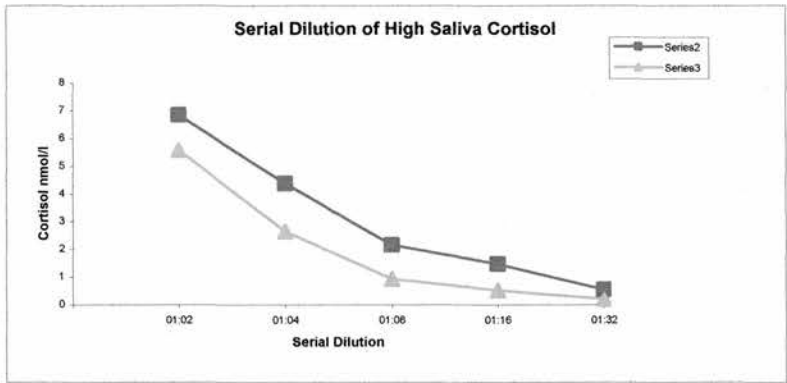
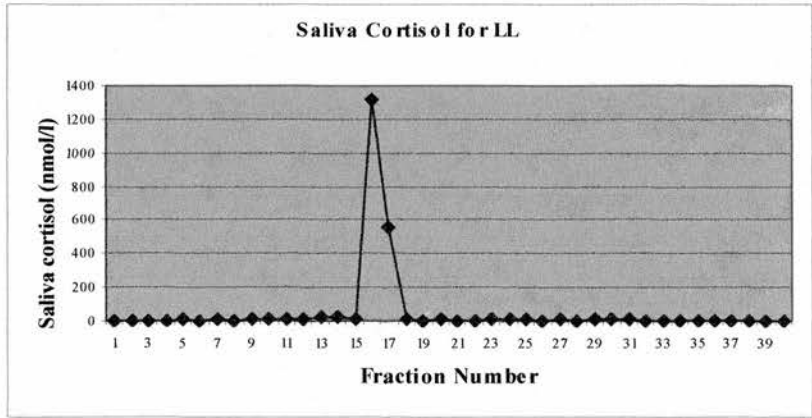


Figure 5-5 HPLC Chromatogram obtained from a saliva sample with a cortisol concentration  $>1000 \text{ nmol L}^{-1}$



### 5.4.5 Mass Spectrometry

The method is described in chapter 3, and confirmed that the molecule present in the sample was cortisol and that it was present in high concentrations.

## 5.5 DISCUSSION

The subjects in this part of the study are representative of sick and pre-term infants in many neonatal units. Most samples were from infants  $\leq 32$  weeks gestational age (mean 28 weeks); 4 samples were from two infants  $\geq 36$  weeks who had infections. Most samples were taken from infants  $\leq 2$  weeks old (mean 4.2 days), with the exception of 8 samples from 8 infants who had BPD (bronchopulmonary dysplasia) who were studied when several weeks old.

The correlation between salivary and total plasma cortisol was good ( $r=0.75$ ) and comparable to those in the literature (Bettendorf *et al.*, 1998; Bober *et al.*, 1988; Francis *et al.*, 1987; Kurihara *et al.*, 1996; Lo *et al.*, 1992; Vining *et al.*, 1983b; Woolston *et al.*, 1983).

Routine clinical laboratories and research laboratories usually measure total plasma cortisol. It is thus relevant to compare salivary cortisol measurements to total plasma cortisol. Salivary cortisol closely follows total plasma cortisol in normal episodic variation, after ACTH and Dexamethasone administration (Laudat *et al.*, 1988; Riad-Fahmy *et al.*, 1983; Walker *et al.*, 1978) and after administration of increasing doses of oral cortisol (Brooks FS, 1984). However, as changes in plasma cortisol-binding capacity can mask either high or low free cortisol levels, the relationship between salivary and total plasma cortisol is less direct than the relationship between salivary and free plasma cortisol. For example, in normal adult subjects total plasma levels up to  $1000 \text{ nmol L}^{-1}$  are associated with normal saliva levels ( $5\text{--}20 \text{ nmol L}^{-1}$ ) implying increased cortisol binding capacity as a cause for their high total plasma cortisol (Lo *et al.*, 1992).

From adult data, there is generally a good correlation between salivary and total plasma cortisol until all the cortisol binding sites in plasma have been saturated. This occurs at a

total plasma level of about 500 nmol L<sup>-1</sup>. Thereafter, an increase in total plasma cortisol leads to a more rapid increase in free cortisol. The slope of the regression line between salivary and total plasma cortisol becomes steeper after this point but the correlation remains close. Thus, instead of a simple straight-line correlation, there are two intersecting lines (Vining *et al.*, 1983b).

The total cortisol binding capacity for plasma in preterm infants is thought to be about 200 nmolL<sup>-1</sup> (Hanna *et al.*, 1997). In this study, saliva-plasma pairs were thus divided into 2 groups, those with total plasma cortisol <200 nmol L<sup>-1</sup> (n=20), and those with total plasma cortisol >200 nmol L<sup>-1</sup> (n=30). The higher levels of salivary and plasma cortisol correlate more closely (r=0.8) than the lower levels (r=0.67). This is consistent with the fact that it is at high levels that the free plasma cortisol predominates, and should relate more directly to the salivary cortisol.

The poorer correlation at values <200 nmol L<sup>-1</sup> total plasma cortisol may be due to inaccurate salivary cortisol estimation. Salivary proteins and enzyme conversion, as discussed below, in conjunction with the inevitable error introduced when working with small volumes of viscous fluid, often diluted and re-analysed, may all have contributed to this. As preterm and sick infants' cortisol binding capacity is often lower but more variable than that of adults (Hanna *et al.*, 1997), it is possible that the total plasma cortisol is a misleading measurement.

There are several potential problems with cortisol measurement in the saliva of preterm and sick infants. While the binding effect of proteins in the saliva of healthy adults is negligible (Walker *et al.*, 1978), it might be greater in preterm and sick infants. Salivary cortisol

binding globulin production may be increased as part of a generalised stress response (Chu and Ekins, 1988; Gabay and Kushner, 1999). Binding proteins may leak into saliva from the plasma through damaged or immature capillary membranes. A resulting increased salivary binding capacity might 'pool' cortisol in the saliva making it disproportionately high. If one or other of these processes were present in the subjects in this study, the effects do not appear to influence the validity of the measure overall. It would be interesting to measure the cortisol binding capacity of the saliva in this population.

Salivary alpha-amylase, mucins and potentially IgA can affect radioimmunoassay accuracy (Fulton *et al.*, 1989; Lantto *et al.*, 1980). Salivary cortisol in infants has been noted to be extremely variable (Francis *et al.*, 1987; Bettendorf *et al.*, 1998). Spurious salivary cortisol levels in preterm and sick infants may be due to high levels of interfering saliva constituents. In this study, it was observed that preterm and sick infants' saliva was often much more viscid than adult saliva. Further studies of the amylase, mucins and IgA in the saliva of preterm and sick infants would be interesting.

In adult saliva, there is evidence of significant  $11\beta$  hydroxysteroid dehydrogenase type 2 activity (Morineau *et al.*, 1997; Vining and McGinley, 1986; Brooks FS, 1984; Katz and Shannon, 1969a; Meulenberg and Hofman, 1990; Vining and McGinley, 1987). This enzyme converts cortisol to cortisone. The activity of this enzyme is very much higher in many organs in the fetus and preterm infant (Stewart *et al.*, 1994; Fujitaka *et al.*, 1997). Poor correlation of salivary and plasma cortisol could result from high activity of  $11\beta$  hydroxysteroid dehydrogenase type 2. The close correlation of salivary and plasma cortisol described in this study implies that this process does not markedly affect the relationship.

The total plasma cortisol levels are in keeping with those in the literature (Barker and Rutter, 1996; Heckmann *et al.*, 1999; Pokela, 1994b). Many studies of preterm or sick infants have found some total plasma cortisol levels  $> 1000\text{nmol L}^{-1}$  (Bourchier and Weston, 1997; Cronin *et al.*, 1993; Economou *et al.*, 1993; Heckmann *et al.*, 1999; Jett *et al.*, 1997; Kari *et al.*, 1996) and some authors have found levels several thousand fold (Barker and Rutter, 1996; Hughes *et al.*, 1987; Pokela, 1994b; Rizvi *et al.*, 1992). Six infants in this study had one sample of total plasma cortisol  $>1000\text{nmol L}^{-1}$ , with the upper end of the range,  $3802\text{ nmol L}^{-1}$ .

The salivary cortisol levels are much higher than those in the literature. Normal adults have morning salivary cortisol means between  $8.7\text{ nmol L}^{-1}$  and  $15.5\text{nmol L}^{-1}$  (Brooks FS, 1984; Hiramatsu, 1981; Laudat *et al.*, 1988; Lo *et al.*, 1992). Tunn found that after intravenous hydrocortisone, the highest total plasma levels of about  $1800\text{nmol L}^{-1}$  were matched with saliva levels of  $482\text{ nmol L}^{-1}$ ,  $414\text{ nmol L}^{-1}$  and  $137\text{ nmol L}^{-1}$  (Tunn *et al.*, 1992). ACTH administration has led to saliva levels up to  $100\text{nmol L}^{-1}$  (Laudat *et al.*, 1988) and  $120\text{ nmol L}^{-1}$  (Vining *et al.*, 1983b). Studies of well term infants in the first days of life have found mean salivary cortisol levels of  $20\text{-}30\text{ nmol L}^{-1}$  (Davis and Emory, 1995a; Magnano *et al.*, 1992; Price *et al.*, 1983; Spangler and Scheubeck, 1993; Klug *et al.*, 2000; Klug *et al.*, 1997). The range in these infants is  $2.5\text{nmol L}^{-1}$ /below detectable range to between  $70\text{ nmol L}^{-1}$  and  $166\text{ nmol L}^{-1}$  (Francis *et al.*, 1987; Gunnar *et al.*, 1995; Kiess *et al.*, 1995; Klug *et al.*, 1997; Kurihara *et al.*, 1996). Levels of salivary cortisol up to  $260\text{ nmol L}^{-1}$  were found in post operative term neonates (Vaughn PR, 1996a).

There are relatively few studies of salivary cortisol in preterm infants. Bettendorph studied pre-term and term infants finding a range of  $0.6\text{-}52.1\text{ nmol L}^{-1}$ , and a mean of  $5.5\text{ nmol L}^{-1}$  with considerable variation (Bettendorf *et al.*, 1998). All, including the 10 preterm infants studied were 'well' and older than the infants in the present study. The authors concluded that salivary cortisol measurement in low birth weight infants was practical and appropriate,

but did not include the assessment of sick or stressed infants. Antonini et al published no absolute values in their study of preterm infants' ( $\geq 34$  weeks gestation) circadian rhythm (Antonini *et al.*, 2000). One publication describes for day 3-7, infants of 26-33 weeks gestational age, a mean salivary cortisol of  $30.2 \text{ nmol L}^{-1}$  paired with mean plasma cortisol of  $372 \text{ nmol L}^{-1}$  (Calixto *et al.*, 2002). An abstract described means of  $5\text{-}54 \text{ nmol L}^{-1}$  depending on whether preterm infants were sick or well. The highest levels quoted are 'median of maximum cortisol' of  $172 \text{ nmol L}^{-1}$  in a group of sick preterm infants (Azcarate *et al.*, 1997).

5 of the 32 infants in the present study had salivary cortisol values  $> 500 \text{ nmol L}^{-1}$ . Their details are displayed in table 5-2. These data illustrate that salivary cortisol measurement identifies those infants who produce extremely high adrenocortical activity in response to significant systemic illness. As seen in the table, gestational age, sex and birth weight do not appear to have influenced the ability to mount this response. These levels of salivary cortisol appear to be higher than those reported for any population.

Table 5-2 Details of infants with salivary cortisol measurement  $>500 \text{ nmol L}^{-1}$

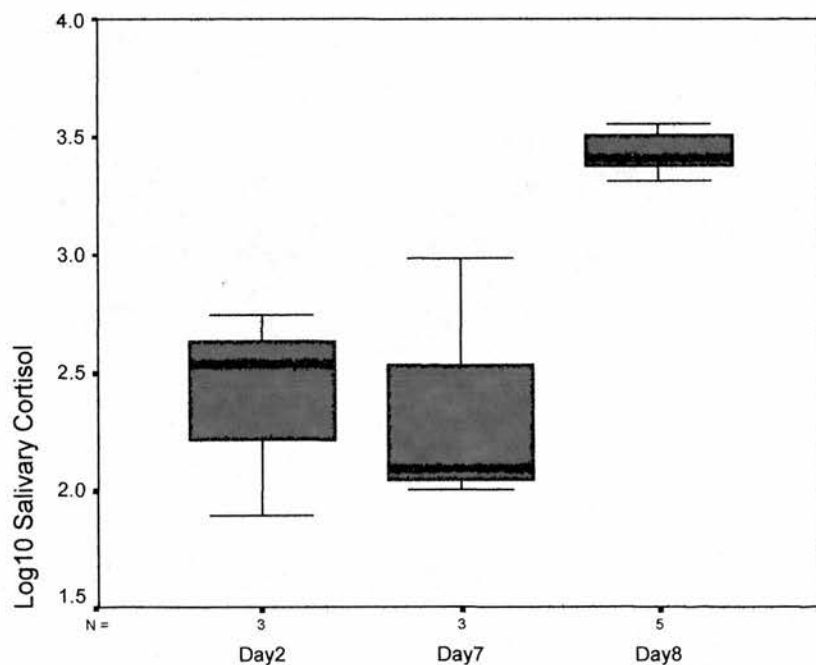
Infant Diagnosis	Total plasma cortisol $\text{nmolL}^{-1}$	Saliva cortisol $\text{nmolL}^{-1}$	Sex F=female M=male	Gestational age (weeks)	Birth weight (kg)	Postnatal age (days)
Sepsis	1118	$>540^*$	F	26	0.595	3
Pneumothorax	1972	$>560^*$	M	28	1.345	2
Meningitis	1386	1159	M	41	3.790	3
Necrotising enterocolitis	2513	2500	M	28	1.210	7
Meningitis	3802	3767	F	36	2.190	2

\* Insufficient volume of sample to re-dilute and re-assay.

As can be seen from table 5-2, a final value was not available for 2 of the samples. This is because despite analysis of more dilute aliquots from the original sample, the value remained above the straight, accurate part of the RIA curve. This highlights one of the technical difficulties with using salivary cortisol as a tool to this population. While some infants, notably those receiving continuous positive airways pressure, had copious saliva, small sample volumes were frequent. In approximately 10 % cases, a final high value for salivary cortisol could not be generated because the value was 'off the curve' and the sample was insufficient for further dilution and analysis. Similarly, difficulty in obtaining a saliva sample from an individual infant in the first place was encountered in approximately 10% of cases. These factors are relevant when considering potential applications of salivary cortisol in sick and preterm newborn infants.

Despite very high salivary cortisol levels, there were many levels at the lower end of the range. 'Well' babies had a tendency to have lower levels than 'sick babies'. It was not the purpose of this study to examine the relationship between severity of illness and cortisol response, but no infant who was regarded as 'sick' had a 'low' cortisol level. Infants who had one or more salivary cortisol level  $>500 \text{ nmol L}^{-1}$  at one time were seen to vary their levels markedly over time depending on clinical condition, so that when they were well, their levels were appropriately lower (fig 5-6).

Figure 5-6 Box plot showing median and range of log10 salivary cortisol (nmol L<sup>-1</sup>). Samples are from a single infant who received CPAP on day 2, was well on day 7 and became ill with NEC on day 8



Median salivary cortisol:

340nmol L<sup>-1</sup>      121 nmol L<sup>-1</sup>      2752 nmol L<sup>-1</sup>

The results show that salivary cortisol continues to reflect plasma concentrations even at very high levels and all very high total plasma cortisol levels are paired with very high salivary cortisol levels. Thus salivary cortisol appears to be a useful measure even in stressed infants. The salivary cortisol medians and upper end of range are higher than any from studies of pre-term infants, or other populations. There does not appear to be a plateau effect with the transfer of cortisol from plasma limiting the concentration obtainable in saliva



It is theoretically possible that cross reactivity of the assay antibodies may have led to falsely high estimates of both plasma and saliva cortisol. See section 3.2.6 and 3.3.4 for cross reactivities.

The only known cross reactivity >5% for a naturally occurring steroid for the saliva cortisol RIA antibody is corticosterone. It has a cross reactivity of 18.6%. (The plasma cortisol RIA antibody had 0.18% cross reactivity with corticosterone.)

Plasma corticosterone levels in newborn infants towards the end of the first week of life are about  $8.3 \pm 1.8 \text{ nmolL}^{-1}$  in term infants (Anand 1992), and  $10 \text{ nmolL}^{-1}$  in preterm infants (estimated from graph) (Doerr et al 1988). Thus, theoretically, the presence of corticosterone in saliva may have led to the overestimation of cortisol in the salivary assay. Only a small percentage of plasma corticosterone (proportional to non-plasma protein bound hormone) would be represented in saliva. There appears to be no literature on salivary corticosterone levels in the neonatal population. Relative to the high concentrations of cortisol measured in sick infants, its expected concentration is not likely to contribute significantly to the final value. In infants with lower cortisol levels, it is possible that its presence contributed more substantially to the final value. This may be a factor explaining the less tight correlation between plasma and salivary cortisol at lower concentrations of both.

The only known cross reactivity >5% for a naturally occurring steroid for the plasma cortisol RIA antibody is 21-deoxycortisol. It has a cross reactivity of 34%. (Unfortunately, there is no data on the cross reactivity with 21-deoxycortisol of the saliva cortisol RIA antibody.) 21-deoxycortisol is formed by the 11-hydroxylation of 17-hydroxyprogesterone. This metabolic pathway is minor in 'normal' steroid metabolism, and basal plasma concentrations range from 0.03 to 0.63 nmol/L in older children and adults. Even after adrenocorticotrophic hormone stimulation, levels are extremely low ( $0.865$  to  $1.50 \text{ nmolL}^{-1}$ ) relative to cortisol. It is classically significantly elevated in cases of 21 hydroxylase deficiency ( $14.5$ - $297 \text{ nmolL}^{-1}$

<sup>1</sup>). As some authors have described raised levels of 17-hydroxyprogesterone in preterm and sick neonates (see appendix 4), it would be logical to expect that these same infants may have elevated levels of 21-deoxycortisol. It is thus theoretically possible that the presence of 21-deoxycortisol in plasma contributed to an overestimation of total plasma cortisol. It is also likely that the sicker or more preterm the infant, the higher the 21-deoxycortisol, and the greater the over-estimation.

Two factors back the conclusion that the cross reactivities did not have a detrimental effect on the functionally accurate estimation of cortisol concentration:

1. Cortisol measurements before and after HPLC gave results within 10% of each other. ie., when the steroids with which the antibodies cross react were removed, the difference was comparable to inter/ intra-assay variation. HPLC and mass spectrometry on 2 samples from 2 infants with high radioimmunoassay-measured levels confirmed that cortisol was demonstrated at high concentrations. It would be ideal to perform HPLC and mass spectrometry on larger numbers of samples in a future study, to strengthen the validation process. The resources required were unfortunately unavailable for this study.
2. Correlation data between salivary and plasma cortisol concentrations were in keeping with those in the literature.

## **5.6 CONCLUSIONS**

- 1) Salivary cortisol levels were wide-ranging and higher than previously reported for any population.
- 2) Saliva and total plasma cortisol levels were closely and significantly correlated.

## 6 Classical circadian rhythm for salivary cortisol in preterm infants.

### 6.1 OVERVIEW

**Background** Circadian cortisol rhythm emerges between 2 and 3 months postnatal age in term infants. It is absent in sick adults. Little is known about its emergence in very preterm infants. This part of the study examines circadian rhythm in cortisol secretion in hospitalised infants born before 30 weeks gestation.

**Design** Prospective longitudinal observational study.

**Patients** 11 infants born before 30 completed weeks of gestation admitted consecutively to the neonatal unit.

**Measurements** Salivary cortisol was measured by highly specific radio-immunoassay on morning and evening samples gathered at weekly intervals until discharge home.

**Results** All values: median 10.0 nmol L<sup>-1</sup>, range <0.5 to 373 nmol L<sup>-1</sup>, interquartile range 4.4-18.0 nmol L<sup>-1</sup>. No infants displayed classical circadian rhythm for 4 weeks or more prior to discharge from hospital. 4 infants displayed a trend towards what may be a 'reverse' circadian pattern. 5 infants >39 weeks post-menstrual age still had no classical circadian rhythm. Taken collectively all values: evening levels were significantly higher than morning ( $p=0.05$ ).

**Conclusion** In hospitalised infants born <30 weeks gestation, adult-type circadian rhythm in salivary cortisol is absent, and in some there may be periods of 'reverse' circadian rhythm.

The task of collecting the samples for this part of the study was shared with a colleague who collected approximately 50% of the samples.

## **6.2 BACKGROUND**

Literature summary and background: See chapter 2.7

## **6.3 METHODS**

### **6.3.1 Subjects**

Consecutive infants born before 30 completed weeks gestation and admitted to the neonatal unit were recruited. For 9 of the infants sampling was commenced before 2 weeks post natal age, and 2 infants were recruited at 6 and 9 postnatal weeks. Details of the 11 infants studied are summarised in Table 6-1. None of the infants received postnatal exogenous steroids. All but two of the infants were exposed to antenatal steroids.

### **6.3.2 Sample collection and analysis**

Saliva was collected as described in chapter 4 and analysed by RIA as described in chapter 3

### **6.3.3 Environment**

In the nursery, lights were dimmed and noise reduced from 2000hrs until 0700hrs. Paired saliva samples were taken at 0900-1000hrs and 2200-2400hrs. The infants had samples taken weekly until they were discharged home.

### **6.3.4 Statistical analysis**

Wilcoxon signed rank test for related samples was used for all analyses on the collective data.

A pattern of salivary cortisol values in circadian rhythm was sought in the subjects' individual graphs as follows:

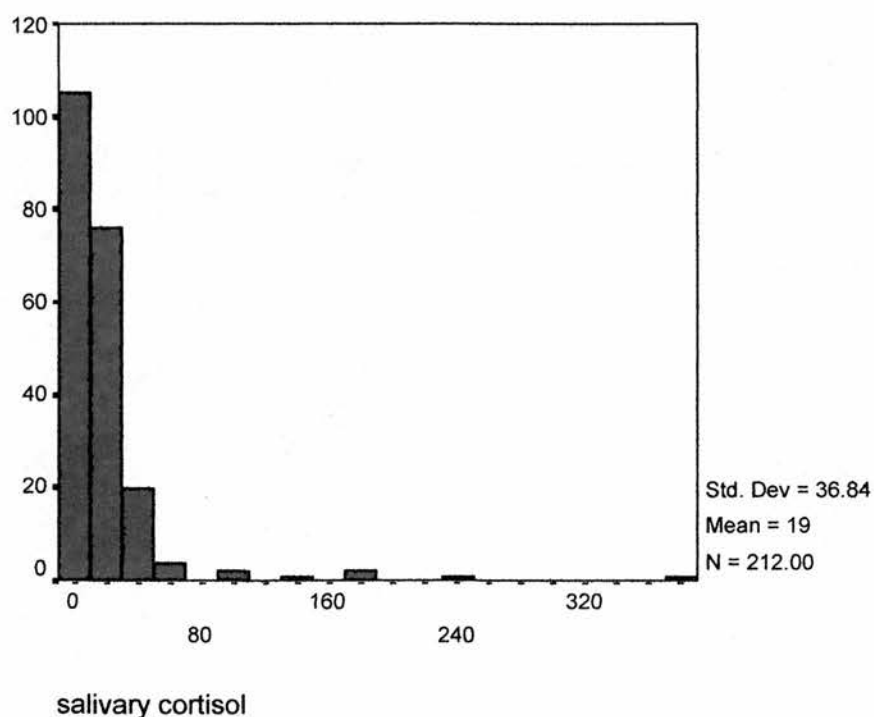
1. Morning > evening value.
2. A drop of  $\geq 40\%$  from morning to evening.
3. The above sustained for  $\geq 4$  weeks.

## 6.4 Results

### 6.4.1 General

There were results for 210 samples comprising 105 pairs of morning and evening samples. For 17 pairs, one or other sample was insufficient or unsuitable for analysis, and therefore the pair was regarded as 'missing data'. The data were positively skewed, and so non-parametric tests were used for collective analyses. The median was  $10.0 \text{ nmol L}^{-1}$  with the range  $<0.5$  to  $373 \text{ nmol L}^{-1}$ .

Figure 6-1 Histogram of salivary cortisol results from all samples ( $\text{nmol L}^{-1}$ )



## 6.4.2 Data for individual babies

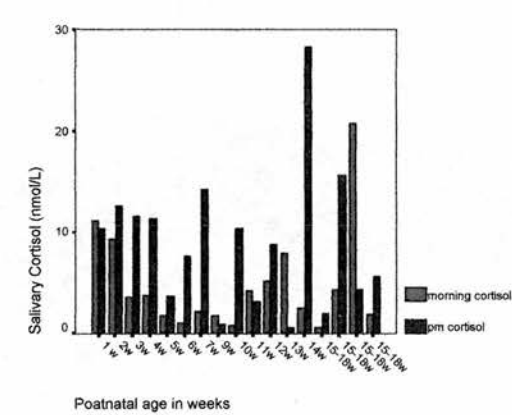
Table 6-1 Characteristics of the infants studied

Infant	GA	Sex	BW gr	Labour	Ante- natal steroids	Race	IVH	Ventilator days	O2 therapy (wks)	Followed from PCA(PNA)	Followed PCA(PNA)(wks)	till Demand feeding(wks)
1	28	f	960	y	n	Oriental- Asian	n	3	16	30(2)	46(18)	9
2	28	f	1255	y	n	Oriental- Asian	gradeIV	13	17	30(2)	46(18)	8.5
3	26	m	980	n	y	Caucasian	grade1	10	18	28(2)	38(12)	11
4	26	m	900	n	y	Indian- Asian	n	8	32	28(2)	38(12)	10
5	27	f	780	y	y	Caucasian	n	22	20+	28(1)	41(14)	11.5
6	28	f	740	n	y	Caucasian	n	33	24+	34(6)	39(11)	10
7	29	m	1115	y	y	Caucasian	n	<1	0.2	30(1)	36(7)	6.2
8	29	m	1360	y	y	Caucasian	n	2	6	31(2)	36.5(7.5)	6.5
9	29	m	1560	y	y	Caucasian	n	5	6	31(2)	36.5(7.5)	6.7
10	29	m	1520	y	y	Caucasian	n	0	24+	30(1)	40(11)	9.5
11	28	m	1370	y	y	Caucasian	n	13	44+	37(9)	43(15)	10.5

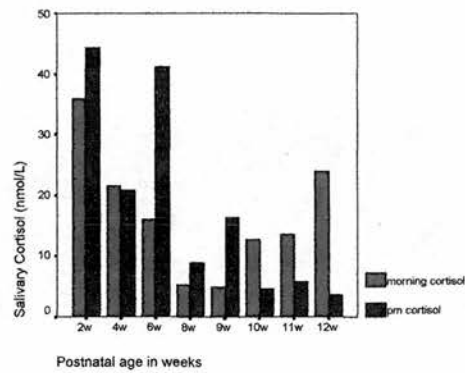
Graphs of morning and evening salivary cortisol for individual babies are shown in Figure 6-2.

No pattern of higher morning levels consistently for 4 weeks was found in any infant, regardless of percentage drop. The closest to the criteria defined for the study was found in infant 4. Morning higher than evening values were sustained for the three consecutive weeks leading up to discharge home, with evening values 63%, 57% and 85% lower than morning values. 5 infants were followed until or past term (>39 completed weeks postmenstrual age) yet still failed to show sustained circadian rhythm.

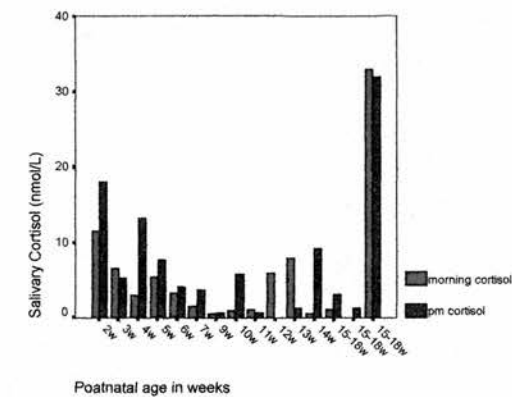
Figure 6-2 Morning and evening salivary cortisol with advancing age in individual infants



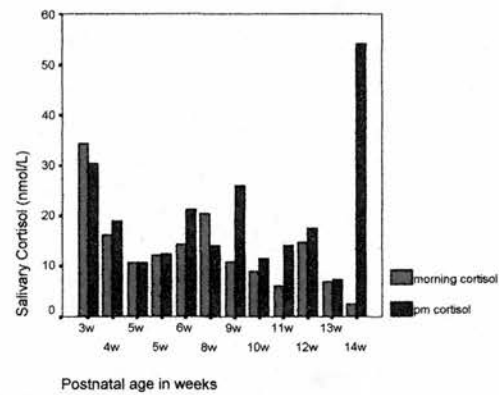
Infant 1



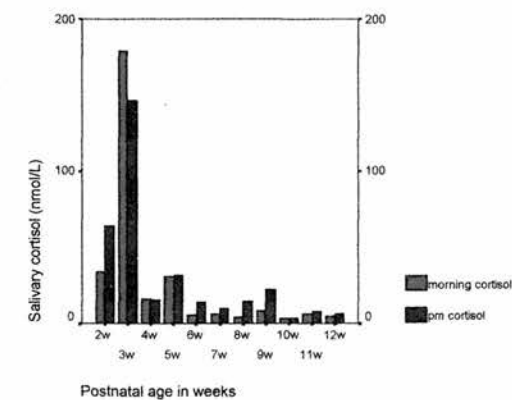
Infant 4



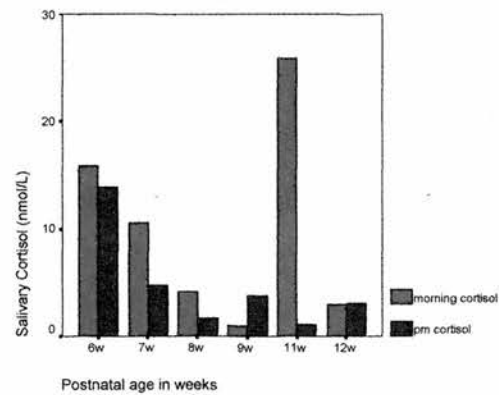
Infant 2



Infant 5



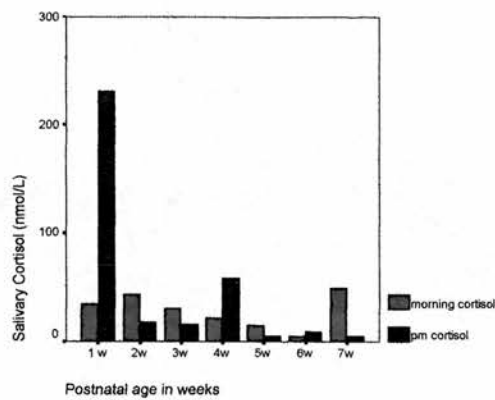
Infant 3



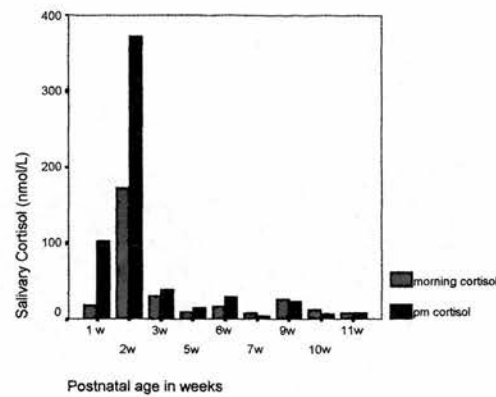
Infant 6



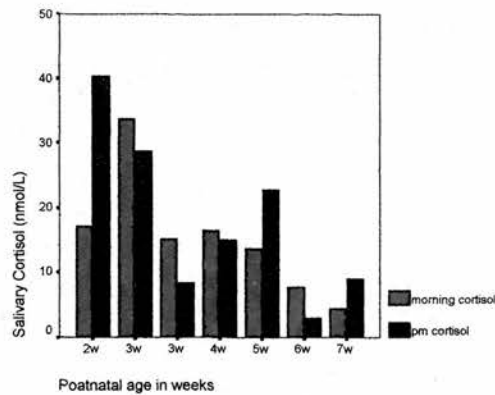
Fig 6-2 continued



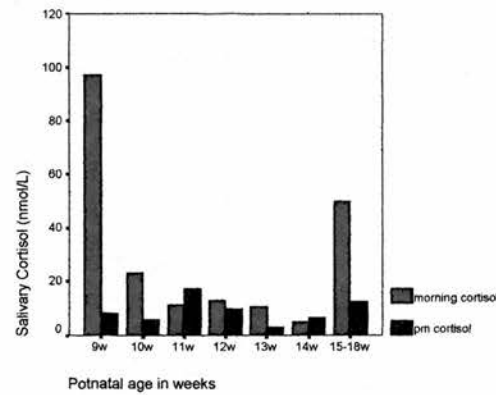
Infant 7



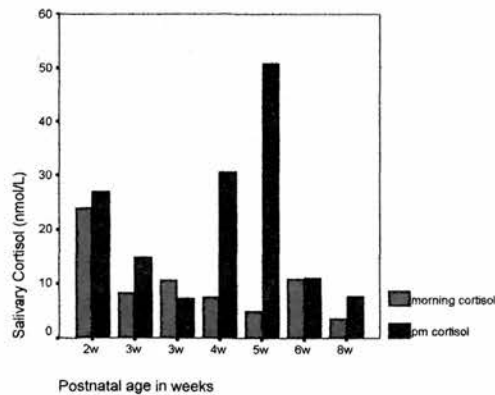
Infant 10



Infant 8



Infant 11



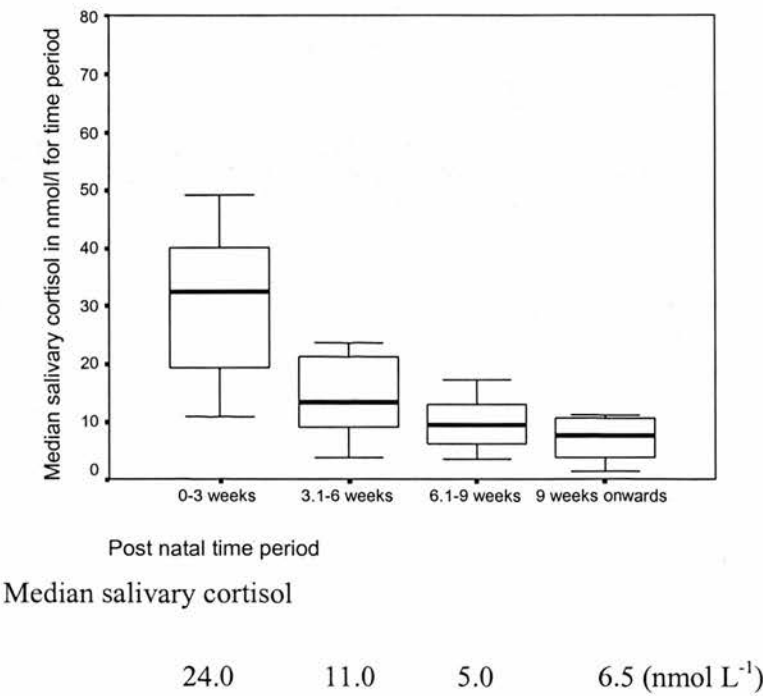
Infant 9

6.4.3 Collective data

Of the 105 pairs of results, 41 had morning higher than evening, and 64 evening higher than morning values. Comparing all the morning samples to all the evening samples, evening samples were found to be higher,  $p=0.025$  using the Wilcoxon signed rank test for related samples. Morning samples: median  $9.3 \text{ nmol L}^{-1}$  (range:  $0\text{-}178 \text{ nmol L}^{-1}$ ), evening samples median  $10.6 \text{ nmol L}^{-1}$  (range:  $0\text{-}373 \text{ nmol L}^{-1}$ ).

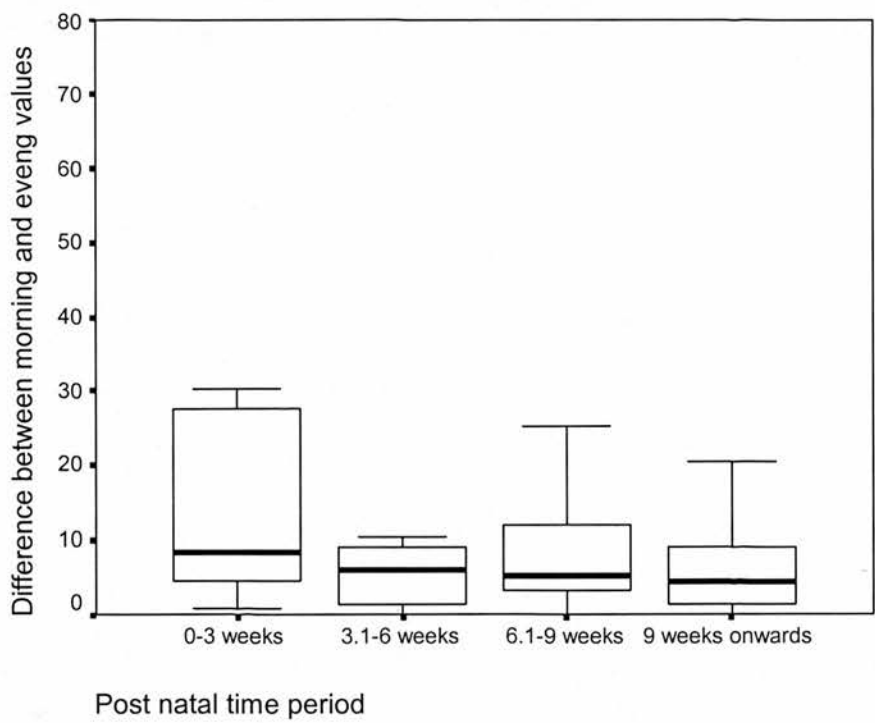
For each infant, the median cortisol value for each three-week postnatal time period was calculated. These values were then grouped with median values from the other infants of the same postnatal age (Figure 6-3). The salivary cortisol in the first 3 weeks of life was found to be significantly higher than in the second, third and fourth 3-week periods ( $p=0.01$ ,  $0.01$  and  $0.03$  respectively).

Figure 6-3 Boxplot of median salivary cortisol values in relation to postnatal age ( $\text{nmol L}^{-1}$ )



There was no significant change in amplitude (i.e. the difference between the two results in each day) with advancing age (Kruskall-Wallis test on the difference,  $p=0.14$ ). See figure 6-4.

Figure 6-4 Amplitude of variation: the difference between the highest and lowest levels of salivary cortisol in each day in relation to postnatal age



## 6.5 Discussion

### 6.5.1 Characterising circadian rhythm

In studies examining circadian rhythm, where multiple samples have been taken over a 24 hour period, mainly in cross sectional studies, ANOVA, conisor analysis, Students t- tests, Goodness of fit and randomness tests have been used (Azcarate AB, 2001; Hindmarsh *et al.*, 1989; Klug *et al.*, 1997; Mantagos *et al.*, 1998), but the data from this study, with paired morning and evening samples, were not suitable for such analysis. Other researchers have used Wilcoxon signed rank or Mann Whitney U tests to compare evening with morning

values (Santiago *et al.*, 1996; Spangler, 1991). Some authors have simply 'looked for' the 'adult type' pattern and amplitude in circadian rhythm. Data from pooled saliva from 8 healthy adult volunteers during the course of this study revealed an evening value 66% less than the morning value, a greater magnitude drop than any published criteria used to detect the presence of circadian rhythm.

Krieger developed a method for characterising circadian rhythm in plasma cortisol in individuals such that the drop from morning to evening value should be at least 35% giving a 14% false positive rate, or 25% drop giving a 22% false positive rate (Krieger *et al.*, 1971). Santiago and Antonini based their analyses of salivary cortisol levels on the coefficient of variation (CV) for the cortisol assay (Antonini *et al.*, 2000; Santiago *et al.*, 1996). For circadian rhythm to be present, the evening level was required to be less than the morning level by three times their intra-assay CV in individuals (22% drop). Applying this method to the present study would also mean that a drop of approximately 22% would be sought in individual infants in this study. Following consultation with a clinical statistician and in light of the above, it was decided that in preference to applying complex statistical analytical tests for longitudinal data, as there were 11 subjects, observation of each infant's individual graph for the following criteria would be used to define the presence of circadian rhythm:

Morning > evening value.

A drop of  $\geq 40\%$  from morning to evening.

The above sustained for  $\geq 4$  weeks.

### 6.5.2 Why there was a lack of adult-type rhythm

#### A. Immature circadian clock.

Despite their presence (Rivkees, 1997), the number or maturation of the cells in the SCN may be inadequate at early gestation to generate and send organised messages to the endocrine systems.

## B. Immature HPA feedback regulation.

The salivary cortisol values are in keeping with cortisol levels published for infants elsewhere (Table 2-1). Presumably because infants were included when they were unwell in the immediate postnatal period, the levels found in the first three weeks of life were significantly higher than in subsequent three-week periods. As illustrated by the charts, there were marked differences in cortisol levels both from week to week, and between the paired samples collected on single days. For the collective data, there was no change in amplitude with advancing postnatal age, whereas term infants are reported to have higher amplitude in cortisol secretion in the first four weeks than beyond (Price *et al.*, 1983). The lack of change in amplitude with postnatal age in this study is consistent with control of cortisol secretion remaining immature in preterm infants even when they are past term for corrected gestational age.

*In utero*, circulating levels of cortisol are low due to inactivation by 11 $\beta$  hydroxysteroid dehydrogenase type 2 in the feto-placental unit. Thus, in the fetus, there is no requirement for inhibition of cortisol secretion by a classical HPA feedback loop. The placental contribution to deactivation of cortisol, however, is removed following birth. Thus the *preterm* infant is exposed to high levels of cortisol that its system may not be mature enough to regulate. This may leave the infant with exaggerated, labile cortisol secretion, possibly hyper-responsive to stress. This is in keeping with the higher and more variable levels of salivary cortisol than those reported in older infants, children and adults. This degree of variability would mask circadian variation.

## C. Antenatal steroid administration.

Most infants in this study were exposed to antenatal steroids (Table 6-1). Some fetal rhythms (movement, heart rate and breathing) are abolished by maternal glucocorticoids (Arduini *et al.*, 1986). It is possible that exposure to large amounts of glucocorticoid at a vulnerable

period might influence the development of circadian rhythm of cortisol secretion. None of the infants received postnatal steroids.

#### D. Lack of circadian 'coaching'.

Instead of circadian rhythms in preterm infants developing at a similar or earlier post-conceptual age than term infants as suggested by Antonini et al (Antonini *et al.*, 2000) they might develop rhythms at a later postconceptional age. Preterm infants have a delay in the appearance of rhythmic 6-sulphatoxymelatonin production compared with term infants, even after correcting for gestational age and length of time at home (Kennaway *et al.*, 1992). Maternal melatonin crosses the placenta freely and there is circadian rhythm in amniotic fluid melatonin (Rivkees and Reppert, 1992). Infants who progress to term are exposed to their mothers' circadian rhythms but preterm infants are denied this input. In theory, then, it might take them longer, i.e. until a greater postconceptional age, to 'organise' their own rhythms.

#### E. Race.

The only infant who came close to displaying circadian rhythm in this study was of Asian origin. Although this is almost certainly due to chance, there are racial differences in other areas of maturation in preterm physiology (Berman *et al.*, 1996). It is interesting to note that Antonini et al (Antonini *et al.*, 2000) who found 'early' circadian rhythm in preterm infants carried out their study in South America. However, these infants were also studied in their home environment, which may have been more conducive to the development of circadian rhythm, and they did not receive antenatal steroids.

#### F. Clinical events reflected by cortisol levels;

Essentially there are no 'preterm controls'. Birth before 37 weeks gestation is abnormal and results in a physiology adapted for intrauterine life being forced to function in an extra-uterine environment. The consequences of prematurity are numerous and include respiratory

insufficiency, vulnerability to infection, hypothermia, hypoglycaemia, and so on. Thus, infants born before 30 weeks gestation will be subject to many physiological stresses. Cortisol secretion in response to these illnesses may simply override and distort any circadian rhythm. This is in keeping with data showing that sick adults in ITU with high cortisol levels lack circadian rhythm (Reincke *et al.*, 1995). In some cases in the present study, clinical events could be related to changes in cortisol levels. For example, Infant 2 (Figure 6-2) developed a respiratory tract infection at 15 postnatal weeks. At this point, the salivary cortisol suddenly jumped from <0.5 and 1.24 to 32.9 and 31.9 nmol L<sup>-1</sup> during the week of the illness.

### 6.5.3 'Reverse' Circadian Rhythm:

Interestingly, although there appeared to be no sustained adult type classical circadian rhythm, a different and unexpected trend became apparent. A sustained pattern of evening > morning levels ('reverse circadian rhythm') was noted over consecutive weeks in 4 of the infants (Table 6-2).

Table 6-2 Details of the salivary cortisol patterns in infants who displayed a trend towards a 'reverse' circadian rhythm.

Subject	Post natal age (weeks, inclusive)	% drop from morning to evening
Infant 1	3-7	69%, 69%, 15%, 86%, 84%
Infant 3	6-9	61%, 33%, 73%, 61%
Infant 2	4-7	78%, 30%, 21%, 60%
Infant 5	9-14	57%, 20%, 57%, 15%, 5%, 95%

While not every percentage difference fulfils the 40% definition, the trend is sustained, the majority of the differences marked and most would be regarded as significant by Santiago, Kreiger and Antonini (Antonini *et al.*, 2000; Krieger *et al.*, 1971; Santiago *et al.*, 1996). The significance of this pattern is unclear. Kiess et al noted (Kiess *et al.*, 1995) that term infants

under the age of 9 months sometimes had evening levels of salivary cortisol 5 times higher than morning levels. It has also been suggested that a reversed rhythm may be associated with chronic stress (Krieger *et al.*, 1971). A similar finding has been reported by Economou, who studied sick preterm infants with respiratory distress syndrome and sick term infants with birth asphyxia or meconium aspiration in the first week of life. His grouped data showed means that were significantly higher at 2000 hours when compared to levels at 0800 hours and 1400 hours (Economou *et al.*, 1993). Preterm infants are frequently exposed to a variety of noxious stimuli, and may become hypersensitive to physical stimuli (Fitzgerald *et al.*, 1989). As the day proceeds, infants may find handling, investigations and environmental stimuli increasingly stressful, and so respond by increasing their cortisol secretion. The concept of 'wind-up', where infants perceive non-noxious stimuli as 'pain' is now widely accepted (Whitfield and Grunau, 2000). This may contribute to 'inappropriate' secretion of high levels of cortisol over the course of the day. A trend towards a 'reverse' circadian rhythm was more prevalent than a true circadian pattern in the subjects in this study.

In this type of study, numbers are inevitably small and sample frequency less than ideal, which can make data analysis and interpretation difficult. For a longitudinal study, however, the numbers are comparable to previous studies and the weekly sampling more frequent. It may be that the 'reverse' findings occurred by chance, but this trend was seen in four infants, and sustained over several weeks in each case. It was a more predominant pattern than the classical adult circadian rhythm. This, along with the collective findings of evening higher than morning values, suggests that further investigation into stress and 'reverse' cortisol rhythm would be warranted.



## **6.6 CONCLUSIONS**

1. There was no adult-type circadian rhythm in any of the 11 infants. All were born at < 30 weeks gestation and 5 were studied to term.
2. A trend towards a 'reverse' circadian rhythm may have been detected with evening values statistically significantly higher than morning values in collective data, and 4 out of 11 infants having periods of consistently higher evening than morning values.

## 7 SALIVARY CORTISOL LEVELS IN 'WELL' INFANTS

### 7.1 OVERVIEW

**Background:** In order to evaluate salivary cortisol as a potential tool for assessing distress in preterm and sick newborn infants, control values are required for comparison.

**Objective:** To generate normal data for salivary cortisol levels in infants with no or minor physical illness and no apparent cause for distress or pain.

**Design:** Combination of cross-sectional and longitudinal data.

**Subjects:** Preterm controls: Gestational age  $\leq 32$  weeks. 2 samples each day.

Day 3 (n=10), Day 7-12 (n=10), Day 13-23 (n=10), Day 24-52 (n=10)

Term controls: Gestational age 37-41 weeks. Single samples. Day 3 (n=20)

Follow-up controls: Term and preterm. Infants who had been ill then recovered. 2

samples each day. Day 7-23 (n=15)

### Results

- 1) For all controls, median :  $19.9 \text{ nmol L}^{-1}$ , range:  $2.5\text{-}102 \text{ nmol L}^{-1}$ , interquartile range  $12.0\text{-}31.5 \text{ nmol L}^{-1}$ .
- 2) There was no significant change in salivary cortisol levels taken from preterm controls across the first 7 weeks of life  $p=0.17$ .
- 3) There was no significant difference between the salivary cortisol levels of term and preterm infants on day 3 of life  $p=0.7$ .
- 4) When they were well, the salivary cortisol levels of infants who had experienced distress earlier in life were not significantly different from the levels of controls with no earlier experience of distress  $p=0.49$ .

## 7.2 BACKGROUND

In order to evaluate salivary cortisol as a potential tool for assessing distress in preterm and sick newborn infants, control values are required for comparison. The aim of this part of the study was to generate normal data for salivary cortisol levels in infants with no or minor physical illness and no apparent cause for distress or pain. Few data exist for saliva levels for the preterm population (Calixto *et al.*, 2000; Antonini *et al.*, 2000; Azcarate *et al.*, 1997; Bettendorf *et al.*, 1998), and plasma levels in the literature for comparison are not from clearly defined groups of infants.

## 7.3 METHODS

### 7.3.1 Study design

Combination of cross-sectional and longitudinal observational study.

### 7.3.2 Sample collection and analysis

Samples of saliva were collected using the method described in chapter 4, and analysed as described in chapter 3. Timing of samples was opportunistic, determined largely by the clinical needs and care-giving procedures being received by the infant. A 'no-handling' period of 1 hour was required prior to each collection of saliva. For preterm infants, and follow-up controls, 3-4 samples were collected from each infant between 6am and midnight on the same study day. The minimum period between collections was 1 hour, and the maximum, 15 hours. 2 saliva samples that yielded results were required for inclusion in analysis. Term infants had a single sample collected.

#### 7.3.2.1 PNA on day of study

Infants were recruited where possible on day 3 of life. (ie >48 hours old). Subsequent samples were taken during the second, third and following weeks of life, to enable a profile of cortisol levels over the neonatal period to be developed. Some infants who fulfilled inclusion criteria but whose parents declined consent initially, were recruited during the

second, third or subsequent weeks of life. 'Follow-up' controls were infants who had been in a 'distress' group, but who had recovered for >48 hours and had no discernible reason to be distressed.

### 7.3.3 Illness severity scores

CRIB and SNAP scores were carried out retrospectively by chart review as described in chapter 2.6, for the time spanning the two samples yielding results on the study day, the eight-hour period prior to this, and the day of birth. Because clinical charts and investigations were not carried out for term controls on the postnatal wards, their scores were presumed to be zero.

### 7.3.4 Subjects

Preterm infants;

- a. Day 3 (n=10)
- b. Day 7-12 (n=10)
- c. Day 13-23 (n=10)
- d. Day 24-52 (n=10)

Term infants;

Day 3 (n=20)

Follow-up controls:

Term and preterm. Infants who had been ill then recovered. Day 7-23 (n=15).

### 7.3.5 Inclusion criteriae

Gestational age  $\leq 32$  weeks for preterm controls or 37-41 weeks for term controls.

Term infants, and day 3 preterm controls: 48-72 hours old

Apgar score at 5 mins  $>7$

BW between 3<sup>rd</sup> and 97<sup>th</sup> centiles

No evidence of, or clinical suspicion of a condition likely to cause distress at time of sampling.

No evidence of, or clinical suspicion of a condition likely to cause distress prior to sampling period, since birth.

No requirement for interventions likely to cause distress, eg mechanical ventilation, CPAP, Lumbar puncture, etc.

No overt signs of behavioural distress as reported by nursing staff, parent or researcher for 1 hour prior to or at time of sampling.

### 7.3.6 Exclusion criteriae

Requirement of incubator O<sub>2</sub>  $>30\%$  in preterm infants

Hypoglycaemia  $< 2.2 \text{ mmol L}^{-1}$

Hypotension ( $<10^{\text{th}}$  centile for BW) (Weindling A.M., 1989)

Treatment with inotropes or postnatal corticosteroid therapy

pH  $< 7.2$  at or in 12 hours prior to time of sampling

IVH

Maternal substance abuse

History of birth asphyxia or Umbilical cord pH  $< 7$

Instrumental delivery or evidence of birth trauma, eg bruising, clavicle or other fracture.

Infants of mothers with significant co-existing medical illness such as diabetes, asthma, adrenal or thyroid disease or epilepsy, were excluded from all groups of the study.

7.4 RESULTS:

7.4.1 General

Salivary cortisol values were obtained from 130 samples from 56 infants in the various control groups.

Where two samples were taken within the study period, the mean of the two was used to represent that infant's 'study-day' result for all analysis.

The distribution of salivary cortisol was skewed to the right so all values were transformed to log 10 for further analysis.

Figure 7-1 Histogram of mean salivary cortisol levels (nmol L<sup>-1</sup>)

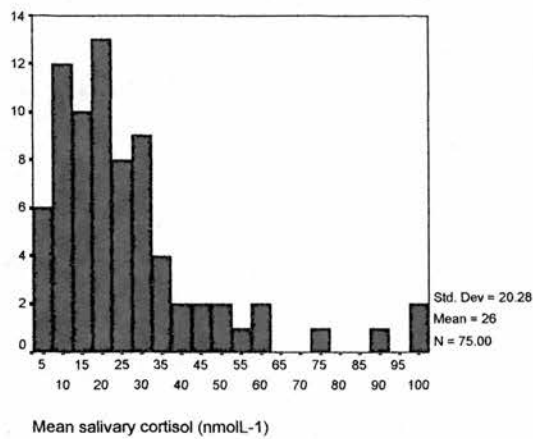
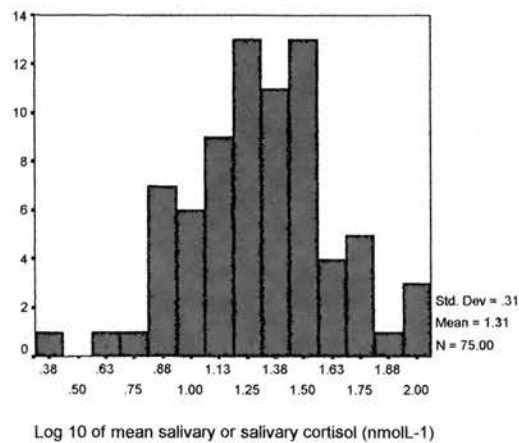


Figure 7-2 Histogram of log10 mean salivary cortisol levels (nmol L<sup>-1</sup>)



7.4.2 Subjects and demographics

Preterm control values were obtained from 21 infants and each post-natal time period studied contained results from 10 of the infants.

Table 7-1 Categorical characteristics of the 21 preterm controls

<b>Total</b>	<b>21</b>
Twin	10
Labour	15
c-section	14
Surfactant	6
Female:male	8:13

Table 7-2 Continuous characteristics of the 21 preterm controls

<b>Characteristic</b>	<b>Mean</b>	<b>Median</b>	<b>Range</b>	<b>SD</b>
GA	30	31	26-32	1.7
BW	1431	1457	705-1955	346
BW centile	44	50	3-90	25
5min apgar	8.4	9	7-10	1.1
1 <sup>st</sup> day SNAP	10.5	11	2-26	6.7
Study day SNAP	2.3	2.0	0-6	1.6
1 <sup>st</sup> day CRIB	1.3	0.	0-7	2.3
Study day CRIB	0.8	0.	0-5	1.4

Figure 7-3 Maternal steroid exposure

<b>Total</b>	<b>21</b>
Multiple maternal steroids	3
Full course maternal steroids	10
Partial course maternal steroids	5
No maternal steroids	3

Table7-3 Pattern of sampling for individual infants over time

Infant	Day3	Day7-12	Day13-23	Day24-52
1	•	•		
2	•			
3	•			
4	•		•	
5	•	•		
6	•			
7	•			
8	•	•		
9	•	•		
10	•	•		
11		•	•	•
12		•	•	•
13		•	•	•
14		•	•	•
15		•	•	•
16			•	•
17			•	•
18			•	•
19			•	
20				•
21				•

Thus, there is a mixture of cross sectional and longitudinal data. Each control time period represents 10 infant-control-days.



#### 7.4.3 Follow-up controls

n=15 (day7-23)

Follow-up CPAP infants: n=5

Follow-up Ventilated infants: n=4

Follow-up NEC infants n=4

Follow-up meningitis infants n=2

#### 7.4.4 Comparison of characteristics of infants in each post-natal time period

In order to detect systematic bias in the characteristics of the infants in the different postnatal time period preterm control groups:

1. A new variable that was the 'mean day of sampling' for each subject was created.
2. For continuous characteristics, a Spearman's correlation between the mean day and the characteristic was sought.
3. For the categorical characteristics, those *with* were compared to those *without* a certain characteristic, within each group separately to see if there was a difference in 'mean day of sampling', using Mann Whitney-U.

Only one of the 15 characteristics (BW, BW Centile, GA, 5 min apgar score, SNAP score for first day of life, SNAP score for study period, CRIB score for first day of life, CRIB score for study period, maternal illness, antenatal steroid exposure, sex, twin pregnancy, labour, foetal illness, caesarean-section) was significantly different between the groups ( $p < 0.05$ ). This would be expected to occur by chance because of multiple analyses and so will be disregarded. (The name of factor was therefore not supplied by statistician who carried out this part of the analysis).

7.4.5 Salivary cortisol levels in control sub-groups

Table 7-4 Salivary cortisol in the control subgroups

nmol L <sup>-1</sup>	Median	Range
Preterm Day 3	25.2	7.5-102.0
Preterm Day 7-12	18.8	6.9-45.0
Preterm Day 13-23	31.1	11.0-49.0
Preterm Day 24-52	14.0	7.6-58.5
Term Day 3	20.4	2.5-97.9
Follow-up Day 7-23	18.8	6.7-49.5

Figure 7-4 Log10 salivary cortisol in the control subgroups (nmol L<sup>-1</sup>)

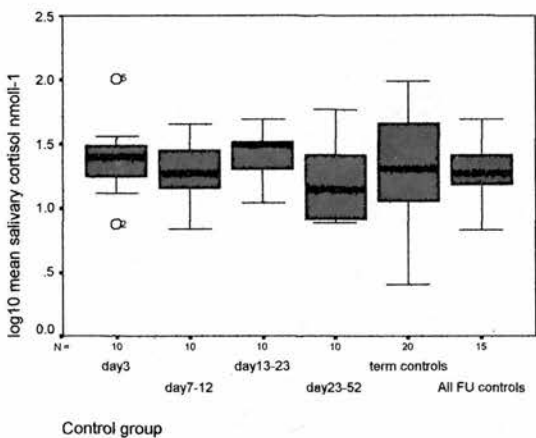
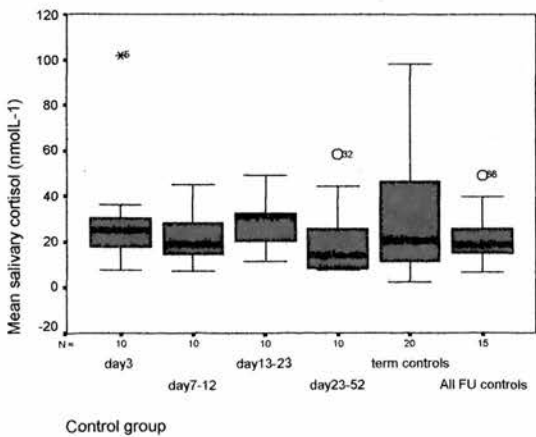


Figure 7-5 Salivary cortisol in the control subgroups (nmol L<sup>-1</sup>)



#### 7.4.6 Statistics: Comparison of salivary cortisol levels in control sub-groups

There was no difference in salivary cortisol between preterm groups across 4 postnatal ages. Mixed model analysis was used because there was a mixture of longitudinal and cross-sectional data, and it was necessary to weight subjects' results appropriately ( $p=0.17$ ).

There was similarly no significant correlation between study day and salivary cortisol result, Spearman's rho correlation,  $p=0.35$ .

There was no difference in salivary cortisol comparing preterm groups to 'follow-up' controls (mixed model analysis  $p=0.49$ ).

There was no difference in salivary cortisol of preterm (day3) compared to term (day3) controls (unpaired t-test on log 10 values  $p=0.7$ ).

#### 7.4.7 Analysis of factors determining/ influencing salivary cortisol level

Correlation (Spearman's rho) was examined for each continuous characteristic at each age group separately. Of the 40 correlations carried out (BW, BW Centile, GA, 5 min apgar score, SNAP score on day of birth, SNAP score on study day, CRIB score on day of birth, CRIB score on study day, maternal illness, antenatal steroid exposure), only antenatal steroid exposure was significant

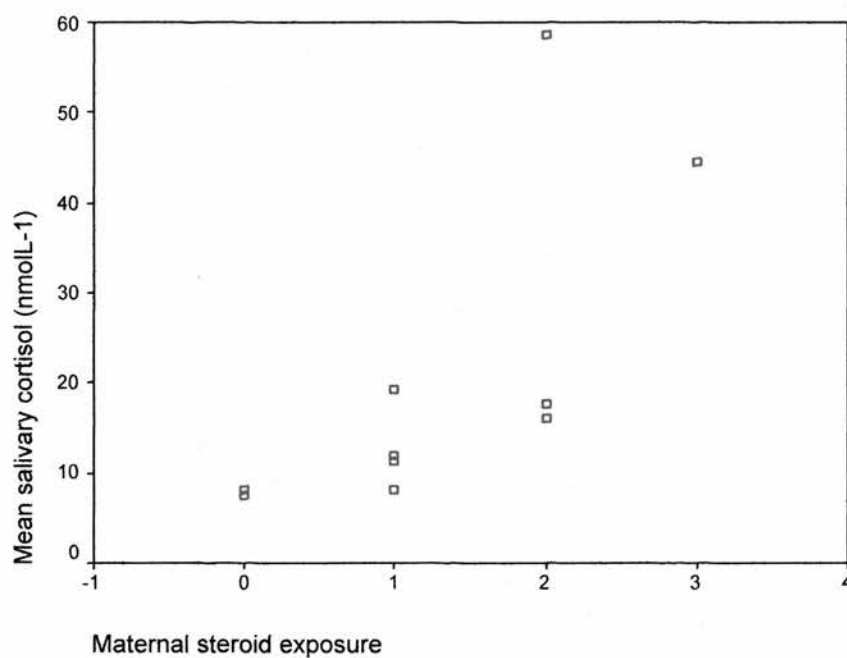
For analysis, antenatal steroid exposure was quantified in a graded fashion, such that;

0= no antenatal steroids, 1= partial course of steroids, 2= full course, 3= multiple courses.

Table 7-5 Correlation coefficients and p values for Spearman's correlation between antenatal steroid exposure and salivary cortisol

Age group	Spearman's Rho correlation coefficient	P value
Day 3 (n=10)	-0.03	0.93
Day7-12 (n=10)	0.31	0.37
Day13-23 (n=10)	0.52	0.13
Day 24-52 (n=10)	0.84	0.02

Figure 7-6 Mean salivary cortisol for PNA 24-52 days in relation to antenatal steroid exposure (nmolL-1)

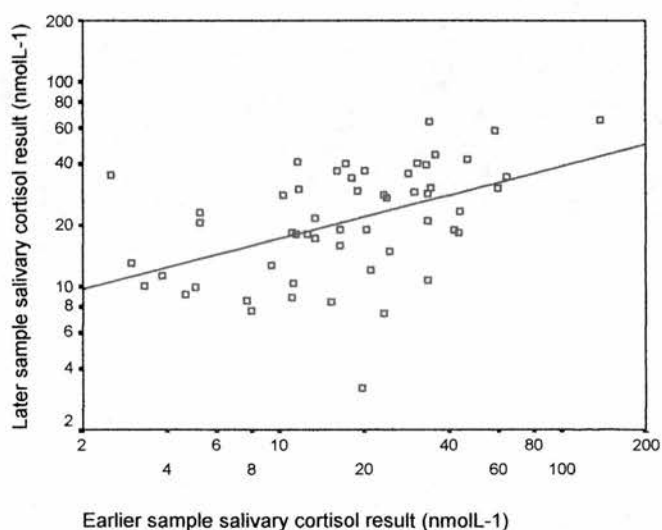


Chi-squared and Mann-Whitney-U tests were carried out for each categorical characteristic at each age group separately. Of the 20 tests (examining sex, twin pregnancy, labour, fetal illness, caesarean section) only one (twin pregnancy) was found to be significant, which would be expected to occur by chance, so will be disregarded.

In conclusion, no significant relationship was found between salivary cortisol level and any perinatal or infant factor, except antenatal steroid exposure in age group 24-52 days, which is examined further in the 'discussion' section of this chapter.

#### 7.4.8 Variation between the two samples taken within each study day

Figure 7-7 Scatterplot of same-day first and second sample salivary cortisol results ( $\text{nmol L}^{-1}$ )



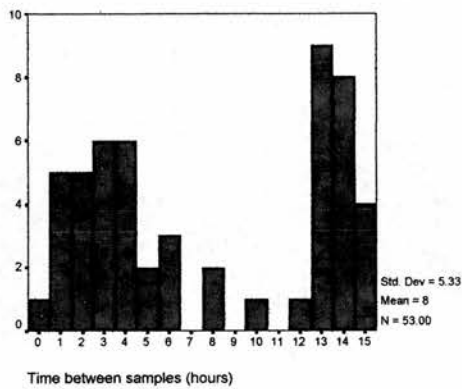
Paired sample t-test showed no difference between the logs of earlier and later in the day salivary cortisol results  $p=0.07$ . Correlation between earlier and later salivary cortisol results significant  $p<0.01$ , Spearman's on cortisol ( $r=0.52$ ), Pearson's on  $\log_{10}$  cortisol ( $r=0.48$ )

There was no relationship between the time between samples and the variation of cortisol levels in those samples: Pearson's correlation of:

1. time between samples
2. difference between cortisol levels expressed as a percentage of the mean of the two levels

not significant ( $p=0.13$ )

Figure 7-8 Histogram of number of hours between samples



Study period ranged from 1-15 hours, mean 7.7, median 7.8, SD 5.3

## 7.5 DISCUSSION

### 7.5.1 Salivary cortisol values in controls

#### 7.5.1.1 General

While it is relatively easy to define a 'normal' or 'control' term new-born infant, the concept of a control preterm infant is more difficult. Premature births are not normal, whether the premature birth has occurred for maternal or infant related reasons. Without medical intervention and support, many premature infants would die or be profoundly damaged. The premature infant's physiology is designed to function within the closely controlled *in-utero* environment. Respiration, digestion, energy homeostasis, waste-product-removal, biochemical equilibrium and temperature regulation are all controlled by maternal/placental function. The preterm infant's physiology may not yet be ready to take on these functions alone. Thus, without medical intervention each one of these realms of physiology becomes abnormal and stressful. Even with medical support and intervention, that their physiology is being forced to function before it is mature is abnormal. Thus, the preterm infant control, is really a 'relative' control, or 'relatively normal' preterm infant. It is a compromise; the nearest to a subject group with whom 'more abnormal' characteristics of sick premature infants can be compared.

#### 7.5.1.2 Distribution

The distribution of salivary cortisol results in this study was skewed to the right with near normal distribution following log<sub>10</sub> transformation. This is in agreement with several other authors studying total plasma cortisol in similar populations (Heckmann *et al.*, 1999; Stevens, 1970; Watterberg and Scott, 1995).



### 7.5.1.3 Timing of samples and variation

It would have been ideal to have samples collected, at a standardised time of, for example, 2 hours apart. However, the constraints of nursing and medical treatments and interventions, along with some samples failing to yield results made this impractical. All samples were collected after a minimum of a 1 hour no-handling period. It was speculated that the effect of handling (Davis and Emory, 1995a) and minor procedures such as venepuncture (Mantagos *et al.*, 1991) would have more of an effect on cortisol levels than the time between samples and the detection of normal hour to hour variation. Only one author in the literature has incorporated a no-handling period into their study on infants in ITU (Heckmann *et al.*, 1999). In the present study, the initial intention was to measure cortisol 4 times in a 24 hour period, but viscosity and volume problems with the samples meant that many samples were unusable. Therefore, the first 2 samples with a single study day that yielded results were used in analysis.

The non-standard sampling regime does not appear to have affected the results. As shown in the 'variation' section of the results, there was no significant difference between salivary cortisol in samples taken at different times of the day from the same infant on the same day. The length of time between samples was not related to the degree of variation between the results.

In acutely ill adults, a single total plasma cortisol value is thought to be sufficient to make a (presumptive) diagnosis of adrenal insufficiency (Grinspoon and Biller, 1994). Jett *et al* also suggest that a single sample of plasma for cortisol levels in preterm infants is adequate to determine adrenocortical function (Jett *et al.*, 1997).

In the term infants, 2 samples would have been ideal. The practicalities of identifying eligible infants, gaining consent from parents, sampling from infants, both following no-handling or feeding times on the postnatal wards made this extremely difficult. The majority of 'normal' infants and mothers are discharged from hospital before or about 48 hours *post-*

*partum*. Therefore, twice the number of subjects as the preterm controls were recruited and sampled once only.

In this study there is no preterm control group born at gestational ages 33-36 weeks. At the beginning of the study, some infants in this group were recruited. It soon became evident that they were going to be a difficult group to follow over time, as many infants were discharged home after 1-2 weeks, or transferred to outlying hospitals. Most of the 'distress' group infants were <32 weeks or term. Thus the decision was made to concentrate on gathering more detailed data from these equivalent control groups to allow more meaningful comparisons.

#### *7.5.1.4 Salivary levels discussed in relation to literature*

##### **Term infants**

The median level of 20.4 nmol L<sup>-1</sup> and range of 2.5-97.9 nmol L<sup>-1</sup> from this study for term, healthy controls sampled on day 3 of life are in keeping with the levels published in the literature. See chapter 2-4 and table 2-3.

##### **Preterm infants**

There are 3 papers and 1 abstract with values for salivary cortisol in preterm infants. The levels are comparable to the median of 23.2 nmol L<sup>-1</sup> and range of 6.9-102 nmol L<sup>-1</sup> from this study.

Calixto found a salivary cortisol mean of 27.6 nmol L<sup>-1</sup> and plasma mean of 372 nmol L<sup>-1</sup> in a group of 48 infants, 3-7 days old, born at 26-33 weeks GA (Calixto *et al.*, 2002).

Antonini *et al* showed salivary cortisol levels of approximately 2-14 nmol L<sup>-1</sup> on a graph from a group of infants born at 31-34 weeks GA, sampled between 2 and 24 post-natal weeks (Antonini *et al.*, 2000).

Azcarate et al found similar levels in sick and well preterm and term infants varying from 1.1-172 nmol L<sup>-1</sup> (Azcarate *et al.*, 1997).

Bettendorph et al found control preterm infants born at gestational age 26-31 weeks, post natal age >2 weeks to have median salivary cortisol levels of 5.5 nmol L<sup>-1</sup>. Term controls at 2 days post-natal age had median level 6.5 nmol L<sup>-1</sup>. These levels are lower than those of the present study, and the other published studies with salivary cortisol levels in the literature for preterm infants (Bettendorf *et al.*, 1998).

### **Estimating total plasma cortisol values from salivary levels for comparison with literature**

The data described in chapter 5 generated a scattergram and regression line and equation which enables the estimation of an equivalent total plasma value for the salivary cortisol levels obtained in this study. Applying the regression equation, equivalent total plasma values were generated for comparison with plasma values in the literature.

Overall regression equation:

$$\text{Salivary cortisol} = 0.87 \times \text{plasma cortisol} - 186.4$$

$$\text{Therefore, Plasma cortisol} = (186.4 + \text{Salivary cortisol}) / 0.87$$

Table 7-6 Estimated equivalent total plasma cortisol values for controls

Cortisol (nmol L <sup>-1</sup> )	Median Salivary	Estimated Median Plasma	Minimum Salivary	Estimated Minimum Plasma	Maximum Salivary	Estimated Maximum Plasma
All preterm controls	23.2	<b>241</b>	6.9	<b>222</b>	102	<b>331</b>
Day3 preterm controls	25.2	<b>243</b>	7.5	<b>223</b>	102	<b>331</b>
Day 3 Term controls	20.4	<b>237</b>	2.5	<b>217</b>	97.9	<b>326</b>

### **Well infants: plasma levels**

The minimum estimated equivalent plasma cortisol for the control infants in this study is 222 nmol L<sup>-1</sup>, which is above the level at which adrenocortical insufficiency would be implied, suggesting that none of the controls infants in the present study displayed 'insufficiency'.

See chapter 2-5.

### **Plasma cortisol levels from studies with comparable, well preterm infants.**

The infants studied by Heckman et al are an appropriate comparison group for the control infants in this study. As well as the levels described, the distribution, with the normalisation following log10 transformation and the clinical characteristics of the infants excluded, are similar. The authors describe a study aiming to establish a reference range for serum cortisol concentrations in preterm infants with a gestational age of less than 30 weeks during the first two weeks of life. Infants were prospectively classified by the following exclusion criteria: surfactant administration, arterial hypotension, acute or uncontrolled infection, ventricular haemorrhage II degrees or above, blood glucose < 2.2 mmol L<sup>-1</sup>, exchange transfusion, stress as a result of any kind of examination or nursing for at least 4 hours before blood sampling. In appropriate for gestational age infants (n = 37, median gestational age 27.7 weeks, median birthweight 1030 g) a reference range was generated: 73-562 nmol L<sup>-1</sup> with a median of 199 nmol L<sup>-1</sup> (Heckmann *et al.*, 1999). This agrees well with the estimated equivalent total plasma cortisol median of 240 nmol L<sup>-1</sup> in preterm controls in the present study.

The estimated median levels of 240 nmol L<sup>-1</sup> in controls from the present study are also similar to the following reports:

1. Thomas et al found basal levels on day 3-4 in 15 preterm well infants of 306 nmol L<sup>-1</sup> (Thomas *et al.*, 1986).

2. Hindmarsh et al found a mean of 294 nmol L<sup>-1</sup> in a group of 10 'unstressed' preterm infants at approximately 3 post natal days (Hindmarsh *et al.*, 1989).
3. In a further study of a group of well preterm infants where SNAP-PE and NTISS scores were used to define illness severity, median total plasma cortisol was found to be 240 nmol L<sup>-1</sup>, <2 weeks PNA, n = 46 (Heckmann *et al.*, 2000).
4. Stevens found a median/ mean (estimated from graph in paper) of 275 nmol L<sup>-1</sup> after the first 24 hours in healthy term infants <1 week old (Stevens, 1970).

Some authors, however, have described lower levels in well preterm infants comparable to the control group in the present study:

1. Arnold et al who found a median cortisol in clinically stable infants born at 25-33 weeks and <9 days old of 176 nmol L<sup>-1</sup> (Arnold *et al.*, 1998).
2. Al Saedi defined reference ranges based on 39 well preterm infants mean 29 weeks, >5 days old. Range 20-680 nmol L<sup>-1</sup>, mean 125+/-107 nmol L<sup>-1</sup> but includes infants up to 8 weeks old (al Saedi *et al.*, 1995).
3. Mean cortisol was described as being 193 nmol L<sup>-1</sup> in premature infants (<38 weeks) in the first month of life (Fujitaka *et al.*, 1997). This is lower than our levels for both term and preterm infants, but does include infants >32 weeks and up to 1 month, which could bring the mean down.

## 7.5.2 Factors potentially influencing salivary cortisol in controls

### 7.5.2.1 Gestational age

The literature referred to is reviewed in detail in appendix 6. In the present study, there was no significant relationship between salivary cortisol and gestational age. This is in agreement with several studies (Huysman *et al.*, 2000; Arnold *et al.*, 1998; Kauppila *et al.*, 1978; Lee *et al.*, 1989; Metzger *et al.*, 1993; Midgley *et al.*, 2001).

When examining the effect of gestational age on cortisol levels, it is logical to account for the potential effect of illness stress on cortisol levels as preterm infants tend to be sicker than more mature infants. In addressing the question of gestational age or commenting on this relationship, many authors do not differentiate between sick and well infants, or do not perform appropriate analysis to account for degree of illness. This may well be the reason that there are several studies describing higher cortisol levels associated with lower GA (Economou *et al.*, 1993; Ng *et al.*, 2000; Nomura, 1997; Rokicki *et al.*, 1990; Scott and Watterberg, 1995).

However, in a group of well infants (clearly defined in paper) studied in the first 2 weeks of life, born at <30 weeks gestation, gestational age was found to be significantly associated with cortisol values (log(10)) so that higher values were obtained in infants born at earlier gestation (Heckmann *et al.*, 1999).

Results from the present study demonstrated no difference in salivary cortisol levels between preterm controls(n=10) and term controls(n=20). Both groups were sampled on day 3. The results are in keeping with findings from 2 other studies making similar comparisons (Azcarate *et al.*, 1997; Kraiem *et al.*, 1985).

Bettendorf, however, found significantly higher salivary cortisol levels in a group of 10 term controls when compared to a group of 10 preterm (26-31 weeks) controls (Bettendorf *et al.*, 1998). The term infants were sampled on day 2, while the preterm infants were 15+ days old. It is quite possible that the term infants' higher cortisol reflected response to delivery. Economou studied infants over the first month of life and found higher control cortisol levels in the preterm 'well' group (GA 33.5 weeks) on day 3 than the term 'well' group. However,

it is not made clear in the paper the criteria for being 'well' in the preterm infants (Economou *et al.*, 1993).

The reason that no relationship between GA and cortisol was detected in the present study may be that the group was too homogenous (GA 26-32 weeks), and did not extend back to 24 weeks GA.

The absence of a detectable difference between salivary cortisol in term and preterm control groups may also be due to type 2 error. If this were the case, it would be likely to be secondary to the relatively small sample size of  $n=10$  in each group.

#### 7.5.2.2 *Postnatal age*

This study was designed originally so that a cohort of 10 infants would be followed sequentially from day 3 over 4-6 weeks, with a result from each infant in each of 4 time periods. Several problems arose with this design:

1. Infants who were recruited initially became unwell, eg with sepsis, necrotising enterocolitis, etc. so were no longer termed 'controls'.
2. Infants who were recruited initially were transferred to outlying hospitals when they were considered to be well enough and so were lost to follow up.
3. Some infants were recruited initially, but the first saliva samples taken were of insufficient volume or too viscous to yield a result.
4. Some infants who fulfilled criteria for recruitment were not entered initially as their parents declined consent until they felt that the infant was 'settled and doing well'. These infants were therefore recruited 'late'.

For these reasons, and described in detail in the 'results' section (Table 7.3), a mixture of cross sectional and longitudinal data was generated, forming 40 'infant study days' from 21 infants. The characteristics of the groups from each postnatal time period did not differ significantly. No significant change in cortisol levels over the first 4-6 weeks of life was found.

Several authors have described a decline in cortisol levels over the first 2 weeks of life in preterm infants (Metzger *et al.*, 1993; Banks *et al.*, 2001a; Kanarek *et al.*, 1988; Kaupila *et al.*, 1978).

In general, most studies do not separate sick from well infants in their analysis of trend in cortisol levels over time and well-defined, reproducible measures of illness are seldom used. Preterm infants are usually sickest during the first days of life. This is likely to make earlier values higher compared to later weeks.

In keeping with this suggestion, Economou found a general decrease in cortisol levels over 15 days. In preterm infants, cortisol levels were higher in the first 5 days than for the rest of first month, with this difference being more pronounced in the sick infants. (Economou *et al.*, 1993). Scott and Watterberg found that the postnatal pattern in cortisol values depended on gestational age. Ill infants more than 27 wk gestational age increased their cortisol values from day 2 to day 6 although cortisol values decreased in well infants. These patterns resulted in a non-significant change over time for these age groups. In contrast, cortisol values significantly decreased from day 2 to day 6 in both well and ill infants that were born at  $\leq 27$  weeks GA (Scott and Watterberg, 1995).

There are probably 2 main reasons why there was no decrease in cortisol levels in controls over postnatal age in this study. Firstly, no samples were taken prior to day 3, so cortisol levels did not detect the effect of the stress of delivery and labour. Secondly, analysis of the



effect of postnatal age was only performed in controls, so stressed infants with higher levels due to illness that occurred earlier in life were excluded. SNAP and CRIB scores are available as clear indicators of illness severity.

In agreement with findings in this study are:

1. Watterberg, Scott, et al. who found no change in cortisol levels in preterm infants over the first week of life, following the first 48 hours (Watterberg *et al.*, 2000).
2. Bettendorf, Albers, et al who found no significant relationship between salivary cortisol levels and postnatal age in preterm infants, who were well and stable (Bettendorf *et al.*, 1998).
3. Arnold et al who found that in infants <9 days old, that there was no association between mean cortisol over 6 hours and post natal age (Arnold *et al.*, 1998).
4. Lee et al who found that following a fall immediately after birth, levels in preterm infants 31-35 weeks GA appeared to be stable over the first week of life. (Lee *et al.*, 1989)
5. Kraiem, who found a rapid fall in cortisol levels after the first few hours following birth, to levels that remained stable for samples taken at 3 and 10 days. This was the pattern for both term and preterm (32+/-2 weeks GA) infants (Kraiem *et al.*, 1985)
6. Ng et al who found no difference in response to hCRH in a group of 14 infants <32 weeks gestation tested on days 7 and 14 (Ng *et al.*, 1997b).

#### 7.5.2.3 Antenatal steroids

The data from this study suggest that antenatal steroid exposure does not significantly suppress salivary cortisol levels from day 3 of life onwards, table 7.5. There was no significant relationship for age groups spanning days 3-23. The significant correlation for day 24-52 of life was one of 40 correlations with salivary cortisol, and the direction of the relationship was opposite to that expected, see fig 7.6, ie. not suppression. It was the advice of the statistician that this result occurred by chance and so should be disregarded. These results are in keeping with the literature on the effect of antenatal steroid therapy on the HPA axis in the newborn period. It suggests that, overall, if suppression occurs, it would seem that it is transient, resolving within the first few days of life. There is no literature describing higher cortisol associated with increasing maternal steroid exposure. See appendix 5.

#### 7.5.2.4 BW

Infants < 3<sup>rd</sup> centile were excluded from any group of the study, as infants with IUGR are known to have different adrenocortical metabolism (Heckmann *et al.*, 1999). In this study, neither birth weight nor birth-weight centile correlated significantly with salivary cortisol values.

#### 7.5.2.5 Sex

It has been suggested that sex (Davis and Emory, 1995b) might contribute to cortisol levels. It is unlikely that the magnitude of influence on cortisol levels would be significant relative to the marked differences between controls and sick infants.

#### 7.5.2.6 Behavioural State

It has been suggested that behavioural state might influence cortisol levels (Anders *et al.*, 1970). In a study of well, stable term and preterm infants, no significant relationship was found between sleep-state or state of arousal and salivary cortisol levels (Bettendorf *et al.*, 1998).

#### 7.5.2.7 Apgar score/ fetal illness

Infants in poor condition at birth or with apgar score less than or equal to 7 at 5 minutes were excluded from the study. This excludes infants with significant asphyxia and/or fetal illness, that might induce a prolonged adrenocortical response lasting into the first week of life that would confound the interpretation of cortisol values related to postnatal distress of identifiable cause. Fetal illness was defined as the presence of any of the following: poor CTG, poor biophysical profile or cord prolapse. Neither apgar score nor fetal illness was related to salivary cortisol levels.

#### 7.5.2.8 Maternal obstetric illness

Maternal obstetric illness was defined as the presence of high blood pressure, *ante-partum* haemorrhage, or infection. Infants of mothers with significant pre-existing or chronic medical illness such as diabetes, epilepsy, or substance abuse were excluded from all groups of the study. There was no difference between the cortisol levels in those infants born to mothers with, compared to those without obstetric maternal illness.

#### 7.5.2.9 Delivery

The effects of delivery on cortisol levels are thought to have subsided by 48 hours PNA (Kauppila *et al.*, 1978). In this study, the presence or absence of labour, and caesarian section were unrelated to cortisol levels.

#### 7.5.2.10 Twin

Those infants born of a twin pregnancy did not have significantly different cortisol levels from those born of singleton pregnancies.

#### 7.5.2.11 Illness severity

There was no correlation between illness severity scores (SNAP or CRIB) from birth or the time of sampling and salivary cortisol levels. This topic is discussed in more detail in the following chapter.

#### 7.5.2.12 Previous pain experience

It is possible that the early pain experience might be associated with a sustained cortisol response, detectable several days or weeks later. This pattern has been observed in patients with burns who have sustained hypercortisolaemia for several weeks following discharge from hospital (Lephart *et al.*, 1987).

Increased neurological plasticity in the neonatal period may mean that exposure to repetitive pain causes permanent or longterm changes. Decreased pain thresholds and altered gene expression in the somatosensory cortex were noted in adult rats that were exposed to repetitive pain in the neonatal period when compared to those that were not. Of particular relevance to the present study, however, no difference was found in corticosterone and ACTH levels following air-puff startle or in pain thresholds in adult rats between those exposed and those not exposed to noxious stimuli in the neonatal period (Anand *et al.*, 1999; Anand, 2000b). Similarly, in this study, 'follow-up control' salivary cortisol levels are not higher than those in 'non-pain exposed' (control) infants.

### 7.5.3 SUMMARY

- 1) For the control group as a whole (preterm, term and follow-up) salivary cortisol levels were: median : 19.9 nmolL<sup>-1</sup>, Range: 2.5-102 nmolL<sup>-1</sup>. The salivary levels and estimated equivalent total plasma cortisol levels are in keeping with the available literature.

- 2) There was no significant change in salivary cortisol levels taken from preterm controls across the first 7 weeks of life  $p=0.17$ .
- 3) There was no significant difference between the salivary cortisol levels from term and preterm infants on day 3 of life  $p=0.7$ .
- 4) When they were well, the salivary cortisol levels of infants who had experienced distress earlier in life were not significantly different from the levels of controls with no earlier experience of distress  $p=0.49$ .
- 5) Salivary cortisol levels in well infants from day 3 onwards are not related to gestational age, post-natal age, exposure to antenatal steroids, sex, birth-weight, birth-weight centile, apgar score at 5 minutes, the presence of maternal obstetric or fetal illness, twin pregnancy, the presence or absence of labour or c-section, or illness severity scored by CRIB and SNAP scores at birth or at the time of saliva sampling.

## 8 Does Salivary Cortisol Reflect Distress in Sick and Preterm New-born Infants?

### 8.1 OVERVIEW

**Background** Salivary cortisol may be raised in sick or pre-term infants subjected to sustained distress.

**Objective** To examine salivary cortisol levels in infants grouped by potentially distressing clinical condition, compared to controls.

**Method** Cortisol was measured in saliva samples from 30 infants with presumably distressing conditions:

- 1) Requiring nasal continuous positive airways pressure (n=10).
- 2) Requiring endotracheal intubation and intermittent positive airways pressure ventilation (n=10).
- 3) With inflammatory conditions; necrotising enterocolitis (n=8) and meningitis (n=2).

Half of the infants (n=15) had follow-up samples taken on a day when they were well, to allow for intra-subject comparison.

### Results

1. Salivary cortisol levels for distressed group as a whole: median: 163.3 nmol L<sup>-1</sup>, range: 22.7-3462 nmol L<sup>-1</sup> interquartile range 107-314.9 nmol L<sup>-1</sup>.
2. Each distress group taken separately had salivary cortisol levels higher than the control group (p<0.01). The infants with meningitis had levels higher than all the other groups taken separately (p=0.01) but there was no difference amongst the other groups.
3. Salivary cortisol levels of infants when 'distressed' were higher than when they were 'well' (follow-up controls) p<0.01.
4. Illness severity assessed by SNAP and CRIB scores at birth or time of cortisol measurement did not account for the differences between the groups.

## 8.2 BACKGROUND

In order to test the hypothesis that salivary cortisol mirrors distress in sick and preterm newborn infants, subjects were grouped according to condition. It was presumed that each condition studied was associated with a certain degree of distress, based on the probable distress that an adult would feel should they be subjected to a similar clinical situation to the infants. The distress groups were graded as follows:

0= no distress (controls)

1= nasal CPAP

2= conventional mechanical ventilation

3= necrotising enterocolitis

4= meningitis

In order to distinguish physiological stress from the presumed distress associated with the diagnosis, illness severity scores were carried out for use in the statistical analysis.

## 8.3 METHODS

### 8.3.1 Sample collection and analysis

Methods as described in chapters 3 and 4

#### 8.3.1.1 *Timing of samples*

Sampling was the same as in the preterm control group described in the previous chapter: opportunistic according to clinical care + no-handling period of 1 hour including 'no feeding'.

### 8.3.1.2 *Study day*

Infants were recruited as soon after diagnosis as possible, usually on the same day as diagnosis.

FU controls: When infants who had been studied as part of a 'distress' group recovered, and were clinically stable >48 hours, with no other apparent cause for distress, saliva was collected on a 'follow-up control' study day.

### 8.3.2 *Illness severity scores*

CRIB and SNAP scores were carried out retrospectively on all the subjects as measures of illness severity. Scores were derived using the tables displayed in chapter 2-6;

For the first 12 (for CRIB) and 24 (for SNAP) hours after birth

For the 8 hour period prior to saliva sampling

For the saliva sampling period

### 8.3.3 *Inclusion criteria*

Minimum age: 48 hours

Apgar score at 5 mins >7

BW between 3<sup>rd</sup> and 97<sup>th</sup> centiles

One main diagnosis/clinical problem/intervention:

- 1) CPAP. Continuous positive airways pressure via nasal device for RDS/pulmonary immaturity/apnoea of prematurity.
- 2) Ventilation. Conventional mechanical ventilation via an endotracheal tube for RDS/pulmonary immaturity; mandatory ventilation or patient triggered ventilation depending on the decision of the infant's clinician. The use of opiate sedation was at the discretion of the clinical team caring for the infant.



- 3) NEC. The presence of a combination of abdominal distension, bilious gastric aspirates, suggestive x-ray changes, and clinical deterioration such that the infant was placed on a 'NEC regime' (triple antibiotics, no milk feeds, total parenteral nutrition, gastric drainage and close clinical monitoring for signs of bowel perforation.)
- 4) Meningitis. Clinical picture leading to lumbar puncture with cerebrospinal fluid changes consistent with acute bacterial meningitis and positive culture of likely organism (group B Streptococcus).

#### 8.3.4 Exclusion criteria

Multiple major problems, eg sepsis + ventilation for RDS, pulmonary haemorrhage + ventilation, NEC + IVH

Hypoglycaemia  $< 2.2 \text{ mmol L}^{-1}$

Hypotension ( $< 10^{\text{th}}$  centile for BW) (Weindling A.M., 1989)

Treatment with inotropes

Postnatal corticosteroid therapy

Maternal substance abuse

History of birth asphyxia

Umbilical cord  $\text{pH} < 7.0$

Instrumental delivery or evidence of birth trauma, eg bruising, clavicle or other fracture.

Infants of mothers with significant co-existing chronic medical illness such as diabetes, asthma, adrenal or thyroid disease or epilepsy, were excluded from all groups of the study.

## 8.4 RESULTS

### 8.4.1 Results: General

Salivary cortisol values were obtained from 60 samples, two samples within each study day from each of the 30 infants in the 'distress' group as a whole. 30 further 'follow-up' results were obtained, two samples from each of 15 of these infants. The mean of the two samples was used to represent that infant's 'study-day' result for all analysis. The distribution of salivary cortisol was skewed to the right, so all mean values were transformed to log 10 for further analysis.

Figure 8-1 Histogram of mean salivary cortisol (nmol L<sup>-1</sup>)

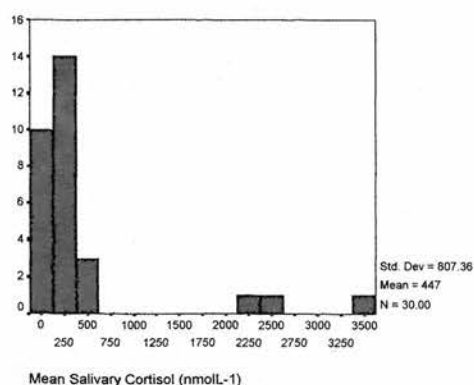
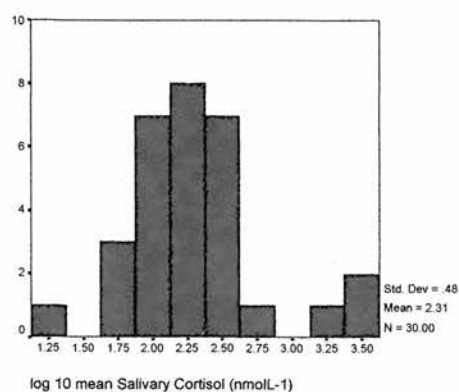


Figure 8-2 Histogram of log<sub>10</sub> mean salivary cortisol (nmol L<sup>-1</sup>)



#### 8.4.2 Subjects and demographics (+ compared to controls)

Table 8-1 Study day for each subject subgroup

	Day of study
Controls (n=10)	3
Cpap (n=10)	3
Vent (n=10)	3-5
Nec (n=8)	3,3,7,8,8,14,15,16
Meningitis (n=2)	3
Follow-up controls	7-23

Table 8-2 Gestational age and birth weight data for subject subgroups

	GA median (mean)	GA range	BW median (mean)	BW range
Controls (day 3)	31(30.9)	29-32	1605(1554)	1040-1850
CPAP	28(28.7)	26-32	1112.5(1163.5)	730-2030
Ventilated	30(29.2)	24-32	1270(1224.7)	593-2035
NEC	28(28.8)	26-34	1065(1222)	595-2131
Meningitis	37(37)	36-38	2990(2990)	2190-3790
All distress	29(28.4)	24-38	1225(1321)	595-3790
Meningitis+NEC	28.5(30.4)	26-38	1277.5(1576)	595-3790
All distress except meningitis	28.5(28.9)	24-34	1192(1192)	595-2131

Table 8-3 Categorical data for subject subgroups

	Sex F:M	Twin	Fetal illness	labour	c- section	Morphine during study	Morphine prior to study period
Controls (n=10)	4:6	5	0	8	5	0	0
CPAP (n=10)	4:6	4	4	4	8	0	2
Ventilated (n=10)	5:5	7	3	5	8	2	8
NEC (n=8)	4:4	2	3	5	5	3	6
Meningitis (n=2)	1:1	0	0	2	0	0	0
All distress	14:16	13	10	16	21	5	16

Table 8-4 Maternal steroid details for subject subgroups

<b>Maternal steroids</b>	Full course	Partial course	Multiple courses	None
Controls (n=10)	6	2	1	1
CPAP (n=10)	4	4	2	0
Ventilated (n=10)	6	2	1	1
NEC (n=8)	4	1	1	2
Meningitis (n=2)	0	0	0	2

#### 8.4.3 Statistics for subject characteristics

Kruskall-Wallis test for multiple comparisons of non-normal data in unrelated groups showed no significant differences between preterm groups:

- a) controls on day 3
- b) CPAP
- c) ventilated
- d) necrotising enterocolitis

for gestational age  $p=0.06$  and BW  $p=0.12$ .

The meningitis infants (who were term) were, as expected, significantly more mature and heavy than the above groups,  $p=0.015$  and  $p=0.026$  for GA and BW respectively.

There were no significant differences for the degree of obstetric maternal illness ( $p=0.5$ ), administration of antenatal steroid ( $p=0.2$ ), 5 minute apgar score ( $p=0.4$ ) or BW centile ( $p=0.74$ ).

Chi squared test showed no difference between the groups for the categorical data; Sex, twin, labour or c-section. Presence of fetal illness was equivalent between the distress groups, but absent in the controls. Fetal illness is defined in section 7.5.2.7.

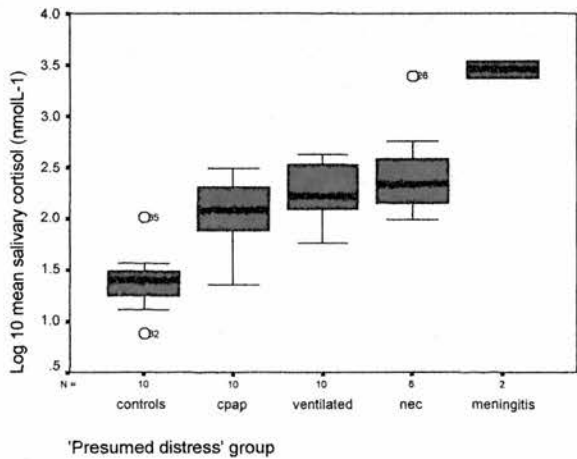
#### 8.4.4 Salivary cortisol levels in 'distress' groups compared to controls

For comparison to controls, the preterm control group day 3 were used, as their cortisol levels were equivalent to all the other control groups (see previous chapter) and the day of sampling was similar to the day of sampling for most of the 'distress' groups.

Table 8-5 Salivary cortisol levels (mean of two samples from one study day) ( $\text{nmol L}^{-1}$ ) in subject subgroups

Salivary cortisol ( $\text{nmol L}^{-1}$ )	Median	Range
Preterm controls Day 3(n=10)	25.2	7.5-102.0
CPAP (n=10)	121.65	22.7-303.6
Ventilated (n=10)	163.1	57.0-416.5
Necrotising enterocolitis (n=8)	218.1	98.2-2469.6
Meningitis (n=2)	2892.1	2322.3-3462.0
Distress group as a whole (n=30)	163.3	22.7-3462.0

Figure 8-3 Log<sub>10</sub> mean salivary cortisol levels in the distress subgroups and control group ( $\text{nmol L}^{-1}$ )



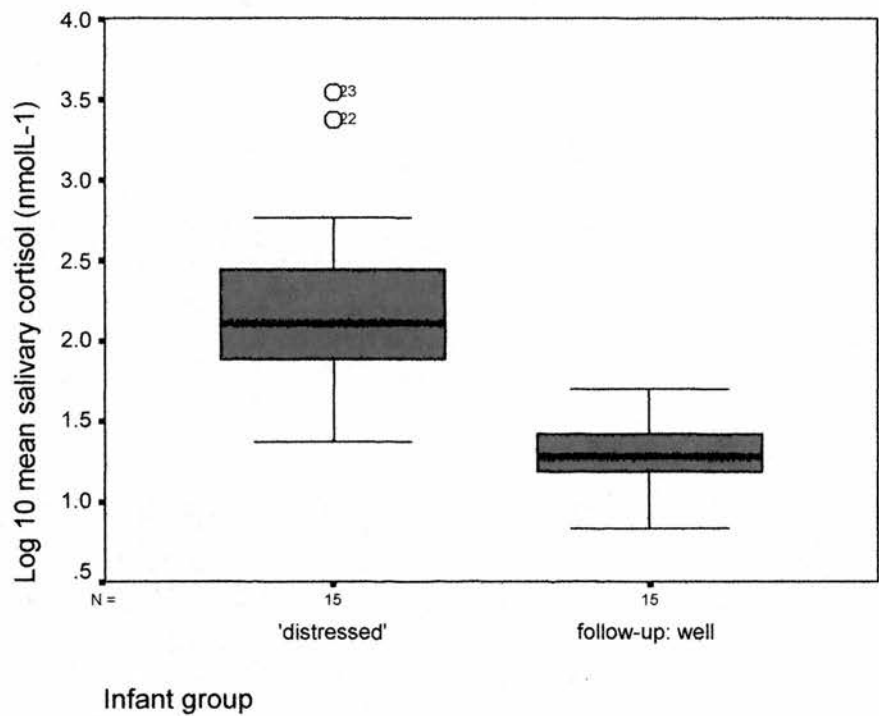
One way ANOVA with Post-Hoc (Turkey HSD) analysis showed that each distress group taken separately had salivary cortisol levels higher than the control group ( $p < 0.01$ ). The infants with meningitis had levels higher than all the other groups taken separately ( $p < 0.01$ ).

There was no difference between the CPAP and ventilated groups( $p=0.7$ ), the ventilated and NEC groups( $p=0.7$ ), or the CPAP and NEC groups( $p=0.1$ ).

8.4.5 Intra-subject comparison of salivary cortisol levels: 'distressed' vs 'well'

Paired sample t-test comparing log 10 mean salivary cortisol of infants when 'distressed' to the same infants when they became 'well' (follow-up controls)  $p<0.01$ .

Figure 8-4 Box plot of log10 salivary cortisol in infants initially in a 'distress' group compared to when clinically well ( $\text{nmol L}^{-1}$ )



**Distressed:** median: 124.8 nmol L<sup>-1</sup> range: 22.7-3462 nmol L<sup>-1</sup>

**Well:** median: 18.8 nmol L<sup>-1</sup> range: 6.7-49.5 nmol L<sup>-1</sup>

#### 8.4.6 Analysis of factors influencing salivary cortisol levels

The General Linear Model (GLM) was used to analyse the effect that potential confounding factors had on the mean salivary cortisol levels. Of particular interest were the illness severity scores. Analysis showed that SNAP and CRIB scores at birth, in the 8 hours prior to or during the study period did not contribute significantly to the cortisol result.

Dependent Variable: LOG 10 Mean Salivary Cortisol. Fixed factor: 'Distress group'

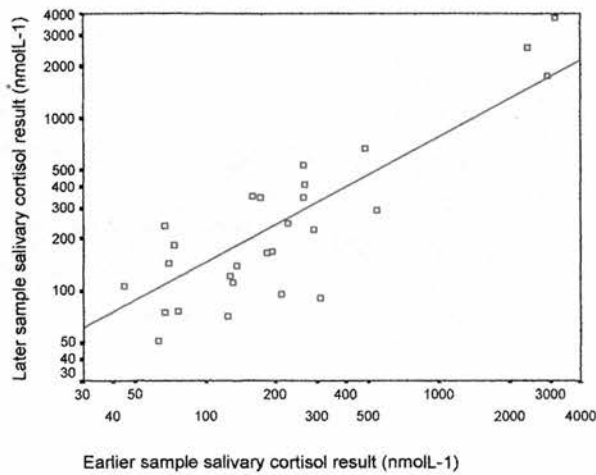
Table 8-6 Significance levels for factors potentially influencing salivary cortisol

Factor	'p' value/ significance
Sex	0.69
BW	0.38
BW Centile	0.52
GA	0.49
5 minute apgar score	0.63
SNAP score 1 <sup>st</sup> 24 hours	0.36
SNAP score 8 hours prior to study	0.90
SNAP score during study period	0.53
CRIB score for 1st 12 hours	0.22
CRIB score 8 hours prior to study	0.06
CRIB score during study period	0.24
Maternal obstetric illness	0.59
Maternal steroids	0.62
Twin	0.55
Fetal illness	0.93
Labour	0.84
C-Section	0.29
Morphine during study	0.46
Morphine prior to study	0.78
PNA (study day)	0.25
'Distress group'	0.02 *



#### 8.4.7 Variation between the two samples taken within each study period

Figure 8-5 Scatterplot of first and second samples taken with study period (nmol L<sup>-1</sup>)



Correlation between earlier and later salivary cortisol results significant ( $p < 0.01$ ) using Pearson's correlation on log10 mean salivary cortisol level:  $r = 0.85$ .

Paired sample t-test showed no difference between log10 means of earlier and later in the day salivary cortisol results ( $p = 0.07$ )

There was no difference in the degree of variation present in the distress group compared to the control groups. Independent samples t-test on log10 difference between the earlier and later cortisol levels in controls compared to pain groups  $p = 0.57$ . (Each control infant was represented only once.)

The time between samples was not significantly related to the cortisol level: correlation between time difference between samples and the degree of variation between those samples ( $[(\text{difference between values} / \text{mean of the 2 samples}) \times 100]$  is not significant, (Pearson's correlation on log10 values:  $r = -0.1$ ,  $p = 0.58$ ).

#### 8.4.8 Illness severity scores

There were significant differences between both of the scores at all of the times measured between the groups,  $p < 0.01$  Kruskal-Wallis test.

Table 8-7 Illness severity scores for 'distress' and control groups

<b>Median (range)</b>	Controls (day 3)	CPAP	Ventilated	NEC	Meningitis
Birth SNAP	6.5(2-16)	14.5(7-25)	15(8-22)	18.5(5-22)	0
Study day SNAP	2.0(0-6)	8(3-15)	14(5-24)	7.0(1-23)	0.5(0-1)
Birth CRIB	0.5(0-3)	2.5(1-6)	3(1-14)	4(0-8)	0
Study day CRIB	0(0-1)	2.5(0-6)	1(0-15)	3(0-8)	0

As both parameters had skewed distributions, Spearman's rank correlation was carried out to explore the relationship between mean salivary cortisol and illness severity scores.

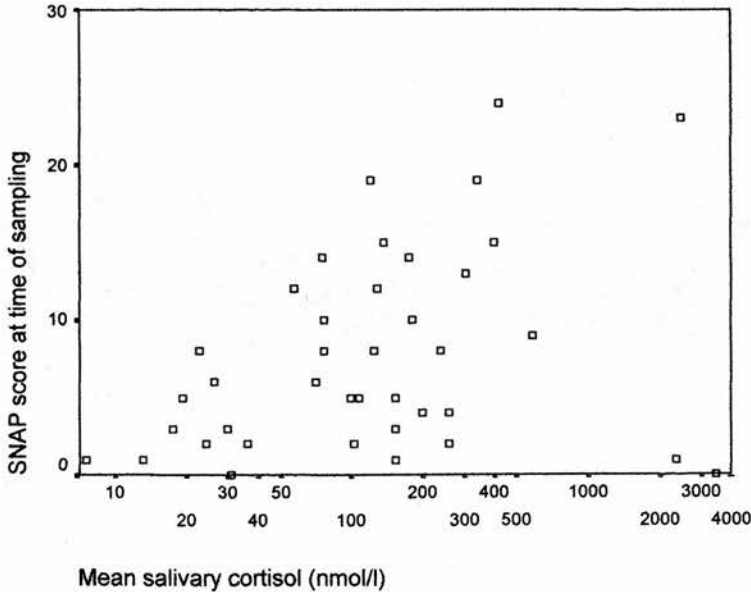
Salivary cortisol from the study period correlated significantly with both scores (CRIB and SNAP) at all times except SNAP score in the 1<sup>st</sup> 24 hours.

Table 8-8 Correlation coefficients and p-values for correlations between salivary cortisol and illness severity scores

<b>Correlation coefficient</b>	<b>Birth SNAP</b>	<b>Pre-study SNAP</b>	<b>Actual study SNAP</b>	<b>Birth CRIB</b>	<b>Pre-study CRIB</b>	<b>Actual study CRIB</b>
<b>Mean Cortisol</b>	0.2	0.4	0.3	0.3	0.4	0.3
<b>p=</b>	<b>0.24</b>	<b>0.03</b>	<b>0.05</b>	<b>0.03</b>	<b>0.01</b>	<b>0.05</b>

The CRIB and SNAP scores correlated with each other at all times, all  $p < 0.01$ , using Spearman's correlation. Scores from different times correlated with each other, all  $p < 0.01$ , using Spearman's correlation.

Figure 8-6 Scatterplot of SNAP score at time of study and mean salivary cortisol (nmol L<sup>-1</sup>)



## 8.5 DISCUSSION

### 8.5.1 Simplification of clinical scenarios for interpretation of results

It is nearly impossible to study intensive care patients in standardised conditions. Most patients requiring intensive care do not have single organ or single system pathology. There are generally many variables at play, both in terms of disease processes and subject responses to those processes. Their interactions are extremely complex and currently poorly understood. In order to simplify the interpretation of results in this study, an attempt was made to standardise patient groups. Patients with more than one main problem were excluded. In addition to this, infants displaying physiological parameters that are known stimuli for cortisol release in themselves, or that might be manifestations of adrenal insufficiency, eg hypotension and hypoglycaemia, were excluded.

While aiding interpretation of results, the drawback of such a design was that patient numbers recruited were reduced. Also, conclusions about the applicability of the results to the neonatal population in general must be to some extent guarded; most infants in neonatal ITU have multisystem pathology, and many suffer hypotension or hypoglycaemia at some stage in their early postnatal course.

### 8.5.2 Timing of samples

As with controls, it would have been ideal to have samples collected at a standardised time of, for example, 2 hrs apart. The constraints described in the controls section were even more restricting in the distressed group as the infants required more intervention on the whole. Hence, sampling was opportunistic. The 'non-standard' sampling regime does not appear to have affected the results. As shown in the 'variation' section of the results, there was no significant difference between salivary cortisol in samples taken at different times of the day from the same infant on the same day. The length of time between samples was not related to the degree of variation between the results.

### 8.5.3 Day of study

There was a significant difference in PNA at sampling time between the distress groups and controls  $p < 0.01$ . An attempt was made to standardise all collections to day 3, 7, etc to allow comparison to controls and to avoid the effect of labour on cortisol levels. Unfortunately, the development of illness was not predictable in this way so it was not always possible to do this. However, salivary cortisol in controls on different postnatal days did not vary. Therefore the decision was made, following consultation with a clinical statistician, to compare all of the 'distress' groups, no matter what day of life their samples were collected, to the day 3 control levels. A substantial number of the 'distress' infants had samples collected on day 3; all 10 CPAP infants, both meningitis infants and 2 NEC infants. All ventilated infants were collected on day 3-5. Study day was also entered as a potential confounder in the GLM analysis. Its contribution was not significant,  $p = 0.25$ . Thus this difference in study day between the groups was not considered to be of importance.

### 8.5.4 Distribution

As with controls, the distribution of salivary cortisol values was highly skewed to the right, and brought to 'normal' by log 10 transformation.

While this is in keeping with the results of many studies, some studies have found 'bimodal' distribution, with a group of infants with low levels of cortisol, consistent with adrenal insufficiency. This is discussed further below.

### 8.5.5 Demographics

The only differences in demographics between the distress groups compared to controls were:

1. **GA.**  $P=0.02$ , which was due to the presence of the 2 term meningitis babies. When the preterm groups were compared ie, the controls, CPAP, ventilated and NEC infants, there were no differences between the groups  $p=0.06$ .
2. **BW.**  $P=0.03$  which was also due to the presence of the 2 term meningitis babies. When the controls, CPAP, ventilated and NEC infants were compared, there was no difference between the groups  $p=0.1$ .

The control infants tended to be more mature and heavier than the distress infants. This reflects the tendency of more immature and lower BW infants to suffer more complications of prematurity. All other population characteristics of the distress infants compared to the control infants were similar.

### 8.5.6 Salivary cortisol papers

There appear to be only two publications with salivary cortisol levels for sick infants:

- 1) Azcarate et al found a maximum level of salivary cortisol in sick preterm infants of  $172 \text{ nmol L}^{-1}$ , which is lower than the upper range of  $300\text{-}3000 \text{ nmol L}^{-1}$  found in the present study (Azcarate *et al.*, 1997). However, only seven sick infants were included in that study.
- 2) Vaughn et al found a salivary cortisol mean of approx  $190 \text{ nmol L}^{-1}$  in term infants in the post-operative period (Vaughn *et al.*, 1996). This level is between the medians found in the present study for ventilated and NEC infants. ( $163 \text{ nmol L}^{-1}$  and  $218 \text{ nmol L}^{-1}$ )

### 8.5.7 Comparison with levels in the literature

As previously discussed, there are few data on salivary cortisol levels in sick or preterm infants. For the comparison of the results from this study with the literature, estimated equivalent total plasma cortisol values were calculated as described in the ‘controls’ chapter (section 7.5.1.4).

Table 8-9 Estimated equivalent total plasma cortisol for ‘distress’ and control groups

Cortisol nmol L <sup>-1</sup>	Median Salivary	Estimated Median Plasma	Minimum Salivary	Estimated Minimum Plasma	Maximum Salivary	Estimated Maximum Plasma
Preterm controls, all PNAs	23.2	<b>241</b>	6.9	<b>222</b>	102	<b>331</b>
Day3 preterm controls	25.2	<b>243</b>	7.5	<b>223</b>	102	<b>331</b>
Term controls	20.4	<b>237</b>	2.5	<b>217</b>	97.9	<b>326</b>
Cpap infants	121.7	<b>354</b>	22.7	<b>240</b>	303.8	<b>563</b>
Ventilated infants	163.1	<b>401</b>	57	<b>279</b>	416.5	<b>692</b>
NEC	218.1	<b>465</b>	98.2	<b>327</b>	2469.6	<b>3053</b>
Meningitis	2892	<b>3586</b>	2322	<b>2883</b>	3462	<b>4193</b>
Above 4 combined	163.3	<b>401</b>	22.7	<b>240</b>	3462	<b>4193</b>

#### 8.5.7.1 Infants with meningitis

Each distress group taken separately had salivary cortisol levels significantly higher than controls. The only subgroup of the distress infants that had levels significantly higher than the other subgroups was the meningitis group.

The salivary cortisol levels of the 2 infants with meningitis were extremely high; higher than reported elsewhere in the literature for saliva. Paired plasma samples were also available,

and these samples comprise some of the highest values discussed in chapter 5. These infants had a serious bacterial infection of a major organ, with a possible generalised or septic component. Neither infant however was physiologically unstable, both having low SNAP and CRIB scores. Neither infant required supplementary O<sub>2</sub>, circulatory support or treatment for hypoglycaemia. As described in the literature review of neuroendocrinology and HPA activation, sepsis, IL1, IL6, and TNF are potent stimuli for cortisol production, with several reports of cortisol levels in septic patients in the many thousands. Ng noted extremely high levels of ACTH and cortisol (1438 nmol L<sup>-1</sup> post CRH) occurred in an infant with bacterial endocarditis (Ng *et al.*, 1997b). In a study of 13 newborn infants (GA 28-40 weeks) with septic shock, endotoxin was recovered from eight infants. Serum cortisol concentration from infants with endotoxemia (2500 nmol L<sup>-1</sup> +/- 1644 nmol L<sup>-1</sup>) was significantly higher than that from infants without endotoxemia (1097 nmol L<sup>-1</sup> +/- 659 nmol L<sup>-1</sup>). The highest single value noted was 6350 nmol L<sup>-1</sup> (Togari *et al.*, 1986). One other infant, who had NEC, in the present study, had salivary cortisol levels in the 1000s. This infant went on to die. It is likely, and in fitting with the clinical picture for the infant, that bacterial sepsis was a contributing factor to the infant's illness.

Thus, the salivary cortisol levels in the infants with meningitis were in keeping with the magnitude of plasma cortisol levels described in serious bacterial infection in the literature, but neither of these infants was shocked, had multi-organ failure or was clinically unstable. Each would presumably have suffered severe pain from meningeal inflammation. Each infant would also have had presumably high levels of circulating IL1, IL6, and TNF. Thus either the pain or the inflammatory mediators could be responsible for the massive cortisol production. With limited data on inflammatory mediators +/- cortisol interactions available for the neonatal population, it seems reasonable to assume that high levels of inflammatory mediators and pain will co-exist in the clinical setting. Thus cortisol released due to



inflammatory mediators would be in keeping with the hypothesis that cortisol levels may mirror pain in this population.

#### 8.5.7.2 Infants receiving nasal CPAP

There do not appear to be any papers that discuss infants requiring nasal CPAP as a distinct group in relation to cortisol levels. Infants in this group would generally have respiratory disease less severe than those requiring intubation and conventional mechanical ventilation. Although the infants do not have a potentially uncomfortable endotracheal tube *in situ*, they do require nasal prongs and a fixation device to keep the prongs *in situ*. There is also the potential for discomfort from having pressurised gas forced into the nose, mucosal irritation or damage from this or the pressure of prongs, and the general restriction of movement and position associated with the treatment. It is conventional not to treat infants who require nasal CPAP with morphine for sedation. This is because it is generally thought that these infants are relatively close to being weaned from all respiratory support, and that morphine may slow down the weaning process. Anecdotally, it has been widely observed that infants receiving nasal CPAP are agitated and may be as distressed as infants receiving conventional ventilation via an endotracheal tube with no sedation. Other researchers have also identified this group of infants as 'potentially distressed' (Debillon *et al.*, 2001).

Results from the present study showed a median salivary cortisol that was significantly higher than controls and lower but not statistically significantly so, than the conventional ventilation group. In contrast, the SNAP scores of the CPAP group were significantly lower than the ventilated group ( $p=0.02$  Kuskall-Wallis test). This is in keeping with the suggestion that distress may be a more important contributory factor to cortisol levels than illness severity.

### 8.5.7.3 Ventilated infants

Infants who received HFOV were not studied. This is because these infants tended to be less mature, more sick and have multi-system pathology. Hypotension was frequently apparent and required treatment with dopamine, and morphine sedation was routine. It was felt that these infants had so many complicated factors potentially influencing cortisol levels that interpretation of their salivary cortisol levels in relation to distress would be extremely difficult. Infants receiving conventional (IPPV) ventilation via an endotracheal tube may suffer distress as a result of the same factors mentioned for the CPAP group, and as listed in the introductory chapter 2-2. Several studies of plasma cortisol in preterm infants separate ventilated infants from other clinical groups. The following studies describe levels in keeping with the estimated equivalent total plasma cortisol median of  $401 \text{ nmol L}^{-1}$  from this study.

#### **Studies with similar cortisol levels for ventilated infants:**

- 1) Reynolds found in 13 infants with non-fatal RDS on day 3 mean levels of  $402 \text{ nmol L}^{-1}$  (Reynolds, 1973).
- 2) Pokela found a median total plasma cortisol of  $420 \text{ nmol L}^{-1}$  in ventilated preterm infants < 1 week old (Pokela, 1994b).
- 3) Ng et al studied 23 preterm infants who were ventilator dependant at 28 days, and found mean levels of  $427 \text{ nmol L}^{-1}$  (Ng *et al.*, 1997a).
- 4) Barker and Rutter studied 47 ventilated preterm infants GA median 28 weeks, at median 15 hours PNA. Prior to diamorphine, the median was  $364 \text{ nmol L}^{-1}$  (Barker and Rutter, 1996).
- 5) Alkalay et al found basal am mean of  $400 \text{ nmol L}^{-1}$  prior to metyrapone in non-adrenal suppressed ventilated infants treated with dexamethasone for CLD (Alkalay *et al.*, 1990).
- 6) In a study of 61 preterm (< 32 gestational weeks) infants, serum cortisol concentrations on day 14 were found to be significantly higher in infants requiring mechanical ventilation than those not ventilated ( $P = 0.014$ ) (Ng *et al.*, 1997c).

In contrast, one group of researchers have found lower levels of cortisol in ventilated infants: Scott & Watterberg found that illness had a significant negative effect on cortisol levels such that the cofactors ventilatory support pattern or "use of surfactant" defined a pattern where cortisol values were lower in infants that had the highest ventilatory requirements or that received surfactant or inotropic support compared with values from those infants who did not have these requirements. Illness severity was assessed by a score based on ventilatory respiratory illness (Scott and Watterberg, 1995). The authors did not discuss the use or effects of sedation in this sicker group with lower cortisol levels. Also, they did not address the potential direct effects of the inotropes or surfactant on cortisol release. Because illness severity was measured in one system only, this may have been a relatively poor measure of illness severity to attempt to relate to cortisol.

The same researchers have consistently described adrenal insufficiency in preterm infants and attempted to relate early cortisol levels with the development of chronic lung disease. In one study, newborns <1500 g birth weight had serum cortisol measured 3 times during days 2 to 7 of life. 'Geometric means' of 125 nmol L<sup>-1</sup> in those who developed CLD and 168 nmolL<sup>-1</sup> in those who did not were found (Watterberg *et al.*, 2000). These levels are lower than those found in this study. In another study, ventilated infants born 24-33 weeks GA, on day 5-7 of life means were in the region of 140 nmol L<sup>-1</sup> (Watterberg and Scott, 1995). This, again is low compared with the ventilated group in the present study.

Some researchers have found no difference in cortisol levels between ventilated and non-ventilated infants. In a study of 21 preterm infants <30 weeks GA, ventilated infants had equivalent basal cortisol levels to non-ventilated infants, and less response to ACTH. The ventilated infants had significantly higher SNAP scores than non-ventilated infants

(Huysman *et al.*, 2000). Ng noted that mechanical ventilation did not seem to influence adrenocortical function (Ng *et al.*, 1997b).

#### 8.5.7.4 Infants with NEC and 'distressed' group as a whole

There appears to be no data directly relating NEC to cortisol levels in the literature. For the sake of comparison of the cortisol levels to the literature, NEC infants in this study are presumed to be equivalent to non-specifically 'sick' infants referred to in many studies of cortisol values in the neonatal population. In the present study the levels in NEC infants were higher but not significantly so than those of the infants in the CPAP or ventilation groups, but significantly higher than controls. Data from this study produce an estimated equivalent total plasma cortisol median for the group as a whole of 401 nmol L<sup>-1</sup> (see table above).

An underlying process in NEC is serious inflammation of the bowel wall. In a proportion of cases, this proceeds to perforation of the bowel wall, and if this is not detected and treated, to peritonitis. An equivalent adult illness with inflammation of the bowel wall and similar potential progression or complications would be appendicitis. The relative area of inflammation is much smaller in appendicitis, but the abdominal pain is notoriously severe. It was therefore presumed that infants with NEC would suffer significant distress. The infants in the NEC group had a wide range of illness severity, from one infant who did not require any ventilatory support, and minimal general intervention, to one who progressed from requiring nasal CPAP to ventilation to collapse and who subsequently died.

Several studies describe cortisol levels in 'sick' infants in keeping with those found in this study:

1. Thomas *et al* found values from day 3-4 of life, in sick preterm infants, mean basal value, 536 nmol L<sup>-1</sup> (Thomas *et al.*, 1986).

2. Hindmarsh et al found a mean of 448 nmol L<sup>-1</sup> in a group of 10 'stressed' preterm infants at about 3 postnatal days. This is comparable to the levels in the present study of 401 nmol L<sup>-1</sup> and 465 nmol L<sup>-1</sup> medians in ventilated and NEC infants respectively (Hindmarsh *et al.*, 1989).
3. Day 3 cortisol mean (360 nmol L<sup>-1</sup>) in preterm 'sick' infants was at the lower end of our estimated plasma total medians for CPAP (354 nmol L<sup>-1</sup>), ventilated (401 nmol L<sup>-1</sup>), and NEC (465 nmol L<sup>-1</sup>) infants (Economou *et al.*, 1993).
4. Hughs et al studied 10 infants <10 days old who were sick and born 24-32 weeks GA. The mean level was 2000 nmol L<sup>-1</sup> on day 3 and maximum was 4000 nmol L<sup>-1</sup>, with wide variation. These levels are in fitting with the meningitis and sickest NEC infants in this study (Hughes *et al.*, 1987).
5. Banks et al found levels at 24 hours to be mean 535 nmol L<sup>-1</sup>, in 314 infants born 24-32 weeks GA (Banks *et al.*, 2001a).
6. Ng et al found basal mean cortisol levels of 396 nmol L<sup>-1</sup> at 7 days PNA in infants <32 weeks (Ng *et al.*, 1997b).
7. Midgley et al found in 22 infants of varying clinical condition, born at 24-31 weeks GA on day 7 median levels of 279 nmol L<sup>-1</sup> (Midgley *et al.*, 2001)s.
8. Merz et al found a basal mean of 346 nmol L<sup>-1</sup> in 34 preterm infants on day 4-7 of life (Merz *et al.*, 1998).

#### **Lower levels in sick infants described in the literature**

Despite the finding that all the groups of infants who were ill, and the sickest infants in this study had higher median cortisol levels than controls, and the extensive literature with similar findings, some studies have described sick preterm infants with low basal cortisol concentrations. The authors of the various studies have suggested a reduced ability in some preterm sick infants to respond adequately to stress during intensive care (Hanna *et al.*, 1997; Kari *et al.*, 1996; Scott and Watterberg, 1995; Arnold *et al.*, 1997; Hingre *et al.*, 1994; Huysman *et al.*, 2000; Korte *et al.*, 1996; Lee *et al.*, 1989; Rokicki *et al.*, 1990; Wittekind *et al.*, 1993). See also appendix 3.

There are many potential reasons for lower levels in the above studies compared with the present study, not least the difficulty in defining standardised groups of infants for comparison. For this study, infants were specifically selected and recruited in order to aid interpretation of the cortisol levels, excluding infants with hypoglycaemia, hypotension or other phenomena that might either induce a large cortisol response, or result from cortisol insufficiency. If such infants had been included, they might have had 'low' levels of cortisol. It was not a primary aim to investigate the presence or absence of cortisol insufficiency in preterm and sick infants. Any subsequent study attempting to relate distress directly to cortisol levels, should include infants with hypotension and hypoglycaemia to examine the extent to which these factors influence the final cortisol level.

The levels in this study may also be higher because there were few infants born at 24 or 25 weeks GA. This is largely as a result of the exclusion criteria regarding multi-pathology. These infants are the population most likely to suffer from adrenal insufficiency. Scott and Watterberg suggest that infants born <27 weeks GA were unable to mount a sufficient cortisol response to illness (Scott and Watterberg, 1995).

Reasons for the disparity between papers on findings of adrenal insufficiency/ response to illness are not obviously attributable to differences in laboratory technique. Reviewing the literature (table 2-4), there are no consistent differences in, for example, use of prior extraction procedure. It also seems unlikely that differences in clinical management in different centres would result in marked differences in cortisol levels in infants eg antenatal steroid dose regimes are standardised.

As discussed in chapter 2.4.3.2, Gunnar proposed that a total plasma cortisol level of 440 nmol L<sup>-1</sup> indicated significant distress in the newborn infant. From the correlation data

described in chapter 5, this plasma level would be equivalent to a salivary cortisol level of approximately 200 nmol L<sup>-1</sup>. However, Gunnar's theory was based on the assumption that cortisol binding capacity in infants was the same as in adults. As detailed in chapter 2.5.8, cortisol binding capacity in newborn, preterm and sick infants is lower; in the region of 200-260 nmol L<sup>-1</sup>. Taking into account the reduced binding capacity of the population, the findings from this study of the lower median of 160 nmol L<sup>-1</sup> for salivary, and estimated equivalent of 400 nmol L<sup>-1</sup> for plasma cortisol in 'distressed' infants are entirely in keeping with Gunnar's theory; the levels from the 'distressed' infants in the present study would be consistent with Gunnar's definition of 'significant distress'.

#### 8.5.7.5 *FU values*

The salivary cortisol levels of infants at the time when they were likely to be distressed was significantly higher than several days later when they were well (FU controls). We have shown that in well infants, there is no such significant decrease in levels that can be attributed to postnatal age alone. Thus, this difference is presumably due to a feature of their illness. Whether this is pain/distress or the physiological stress of illness severity is discussed below (SNAP scores etc). The FU control values are not higher than salivary cortisol levels from infants who have not experienced previous distress of a similar magnitude. As discussed previously (chapter 7.5.2), it is possible that the earlier experience of pain would be associated with a sustained cortisol response, detectable several days or weeks later. This study did not confirm this hypothesis.

#### 8.5.8 Factors determining salivary cortisol

The general linear model was used to assess the contribution of various factors to the final salivary cortisol result. 'Distress' group was graded 0=controls, 1=CPAP, 2=ventilated,



3=NEC, 4=meningitis. 21 factors were analysed: 'distress' group, post-natal age, sex, BW, GA, 5 minute apgar score, BW centile, maternal obstetric illness, maternal steroids, twin, fetal illness, labour, caesarian-section, morphine received prior to study period, morphine received during study period, birth SNAP score, birth CRIB score, actual study SNAP score, actual study CRIB score, SNAP score for 8 hours prior to study, CRIB score for 8 hours prior to study. The only factor that had a significant contribution to the salivary cortisol was 'presumed distress' group  $p=0.02$ .

#### *8.5.8.1 Influence of gestational age in pain infants*

There was no relationship between GA and cortisol levels in the 'sick' infants in this study, taken as a group (CPAP+Ventilated+NEC+meningitis) (general linear model,  $p>0.5$ ). This implies that immature infants have adequate capacity to mount a cortisol response to stress. In one study, sick preterm infants had higher cortisol levels than sick term infants (Economou *et al.*, 1993). Several authors have described more premature infants as having higher cortisol values than more mature (Ng *et al.*, 2000; Nomura, 1997; Rokicki *et al.*, 1990; Scott and Watterberg, 1995). This is probably because preterm infants tend to be sicker than more mature infants. As discussed in the 'controls' chapter, and appendix 6, there are as many or more authors who have failed to describe a clear relationship between GA and cortisol level.

#### *8.5.8.2 Influence of Morphine on cortisol levels.*

Several authors have shown that opiates can decrease plasma cortisol levels (Barker and Rutter, 1996; Pokela, 1993; Vaughn PR, 1996b). None of the control group received morphine. Morphine received during the study period by 5 of the 30 'distress' infants did not influence the final salivary cortisol result. Prior exposure to opiates did not influence cortisol



levels either. It may be that within an individual, the use of opiate will induce a significant reduction in cortisol level, but with the spread of values in the various clinical groups, this effect is statistically lost.

The other factors, BW, PNA, BW centile, apgar score at 5 mins, etc that did not have a significant effect on the cortisol levels in distressed infants are discussed in detail in the 'controls' chapter (7).

#### 8.5.9 Illness severity

There were significant, if relatively weak, correlations between salivary cortisol and all of the scores performed with the exception of SNAP score for the first 24 hours after birth ( $p=0.24$ ). These correlations were stronger when all of the control infants (not just day 3 preterm controls) were included in the analysis. GLM showed that 'presumed distress group' was more closely related to salivary cortisol ( $p=0.02$ ) with illness severity having an insignificant contribution ( $p>0.05$ ).

Adult ITU patients have higher total plasma cortisol than controls, and there is a correlation between the levels, severity of illness and mortality (Jurney *et al.*, 1987; Reincke *et al.*, 1995). Two other studies in the neonatal population have found a positive relationship between illness severity score and cortisol level:

1. In a study of 314 infants born at gestational ages 24-32, CRIB scores were positively associated with cortisol level on days 1 and 3 through 7 (Banks *et al.*, 2001b).
2. Barker and Rutter studied cortisol and adrenaline responses in 47 ventilated preterm infants who were judged clinically to require sedation. Stress hormone concentrations were significantly correlated with severity of illness prior to and after sedation, assessed using the arterial: alveolar oxygen partial pressure ratio. Noradrenaline showed the strongest correlation, but the correlation coefficient between cortisol and arterial:

alveolar oxygen partial pressure ratio was significant at  $p < 0.05$ ,  $r = 0.33$  (Barker and Rutter, 1996). These figures are very similar to the correlation data between both SNAP and CRIB scores at the time of sampling in the present study, and salivary cortisol.

Several studies of the newborn population relating illness severity scores to cortisol levels failed to demonstrate a similar positive relationship (Arnold *et al.*, 1998; Hanna *et al.*, 1997; Heckmann *et al.*, 2000; Huysman *et al.*, 2000; Jett *et al.*, 1997; Scott and Watterberg, 1995). See appendix 1 for details of these studies. The present study measured salivary cortisol, which may be a more biologically relevant measure than plasma cortisol. This may have enabled a positive correlation with illness severity to emerge that would not had total plasma cortisol been measured.

This study may also have been able to demonstrate a significant relationship between illness severity and cortisol because of the recruitment protocol. This excluded infants with overt signs of cortisol insufficiency/stimuli for cortisol release in order to simplify interpretation of results. Thus, some sick infants with low cortisol may have been excluded. The debate about the existence of adrenocortical insufficiency in preterm and sick infants, its magnitude and significance remains unresolved. Whether this is actually important regarding the present study is not clear; most studies attempting to relate adrenocortical function to hypotension, one of the most significant and dramatic signs of adrenocortical insufficiency, have failed to identify a direct relationship. The possibility of a subgroup of infants with adrenocortical insufficiency remains, but further research is required to characterise and identify it.

In summary, illness severity, assessed by deviation from normal physiology, in the form of the SNAP and CRIB scores, does not appear to be related to cortisol as closely as 'presumed

distress group'. This holds true even when the extremely high cortisol values of the infants with meningitis (and low SNAP scores) are removed from the GLM.

This may be because either;

1. While they are good measures, SNAP and CRIB scores are not ideal measures of physiological stress. Scoring may become more predictive of cortisol levels if the contribution of inflammatory mediators is somehow incorporated into or partnered with existing scores or
2. Because distress *is* a significant entity distinct from physiological stress and is a potent stimulus for cortisol release.

## 8.6 SUMMARY AND CONCLUSIONS

The results from this study suggest that the degree of distress that an infant is presumed to suffer, based on his or her diagnosis, is more closely related to salivary cortisol level than any other factor analysed, including scored illness severity. There are data relating distressing stimuli such as circumcision and venepuncture to raised cortisol levels in newborn infants, and a substantial literature confirming the relationship between psychological 'distress' and cortisol levels in medically well adults. Despite this evidence, researchers working with medically sick infants have not generally reported or discussed the effects of pain or distress on cortisol levels in their patients.

In this study, 'sustained distress' has been identified and examined as an important contributory factor to the level of salivary cortisol in preterm and sick newborn infants. Since this study was carried out, a recent publication has described a behavioural tool for the assessment of sustained distress in the newborn infant (Debillon *et al.*, 2001). The use of such a tool, in conjunction with physiological stress measures such as the SNAP score, the measurement of inflammatory mediators, and an indicator of free, biologically active cortisol may further tease out the nature of the relationship between physiological and psychological distress and cortisol in the sick newborn infant. It seems sensible that this next stage of research should include infants with possible signs of adrenal insufficiency, and with multiple pathology, so including more infants with gestational ages in the 24-26 week range. Investigation into the effect that distress-alleviating measures have on salivary cortisol in sick and preterm infants with continuing pain or distress would also be helpful.

The simple findings from this study remain, however, that salivary cortisol levels were raised in infants with physiological illness that is almost certainly associated with sustained

distress. This is consistent with the hypothesis that salivary cortisol is a potential mirror of sustained distress in the preterm and sick newborn infant. Salivary cortisol thus has potential as an important component in the assessment of sustained distress, and as a tool for monitoring the effects of distress-alleviating measures.

## 9 APPENDICES

These appendices are intended to pull together as much information as possible on specific areas of interest. They refer to subjects that are covered in the literature review chapters and the discussion sections of the later chapters.

### 9.1 Appendix 1 Studies with data on the relationship between cortisol and degree of illness

#### Studies finding no difference in cortisol levels between well and sick infants or no correlation between clinical features and cortisol

In a study of term infants in the first 3 days of life, Gutai found no difference between control cortisol levels and those from sick infants (suffering from asphyxia, RDS, torsion of testis and sepsis) (Gutai *et al.*, 1972)

In a study of 13 well and 9 sick preterm infants, cortisol values were not different between the sick and healthy infants and were similar to full-term values. The authors did, however describe higher steroid precursors (17 OH-P and 11-deoxycortisol) in the sicker infants. The infants were 31-35 weeks GA, and the 'sick' infants had 'moderate to severe RDS' (Lee *et al.*, 1989).

Ng noted that mechanical ventilation did not seem to influence adrenocortical function, but that extremely high levels of ACTH and cortisol ( $1438 \text{ nmol L}^{-1}$  post CRH) occurred in an infant with bacterial endocarditis (Ng *et al.*, 1997b).

In a study of 26 infants born at 25-30 weeks GA, over 16 weeks (total 95 samples), Arnold *et al* found that plasma cortisol was not influenced by the clinical state. 'Unwell' was defined as requiring 25% or more  $\text{O}_2$ , IV fluids or mechanical ventilation, however, implying that the 'unwell' infants may not have been particularly 'sick' (Arnold *et al.*, 1997).

In a prospective study, serum total cortisol and CBG were measured in single blood samples from 31 ELBW infants, with GA < 28 weeks, in the first 8 days of life. Severity of illness was assessed using the Score for Neonatal Acute Physiology Perinatal Extension (SNAP-PE). There was no significant correlation between serum total cortisol and severity of illness. Cortisol was not significantly different in the 7 of 31 infants who died. However, the samples were taken at varying post natal ages, the samples were random, and numbered only 31 in total, details of the laboratory methodology are not given, and illness scores were at birth and not at the time of sampling (Hanna *et al.*, 1997).

Thomas *et al* found that there was no significant difference in basal plasma cortisol concentrations on day 3-4 PNA between sick preterm infants (mean  $536 \text{ nmol L}^{-1}$ ) and healthy preterm infants (mean  $306 \text{ nmol L}^{-1}$ ), although they were highest in preterm ill infants (Thomas *et al.*, 1986). The group of sick infants included sepsis, asphyxia, RDS, post-op.

Arnold *et al* performed SNAP scores on the day of cortisol measurement in 26 infants. All infants were < 9 days old, one 24 hours, 5 on day 2. There was no correlation between the SNAP score and mean cortisol over the 6 hr study period. 21 of the 26 infants had mild illness, with scores 0-9. The remaining 5 had moderate illness with scores of 10-19 (Arnold *et al.*, 1998). Thus, this group did not include a wide spectrum of illness severity, and had only a small number of the more moderately sick infants. This may account for the failure to find a relationship between illness severity and cortisol levels.

In a study of 25 infants, mean GA 28 weeks, over the first 8 weeks of life, at none of the postnatal ages studied was plasma cortisol found to be influenced by the degree of illness. (Wittekind *et al.*, 1993).

A study of 12 ventilated premature infants on day 2 found no relationship between plasma cortisol or urinary noradrenaline and degree of respiratory disease severity (Wahlig *et al.*, 1994). This may be due to the confounding effects of the continuing influence of labour +/- delivery on cortisol on day 2.

In a study of salivary cortisol levels, no difference was found between levels in sick vs well infants (Azcarate *et al.*, 1997). There were, however only 7 infants in each group, and no definition of well or sick was given.

Jett *et al* performed multiple sampling over a 6 hour period in 14 infants <28 weeks GA on day 4—6 of life, and SNAP-PE scores for the first 24 hours and on day of sampling. No relationship was found between mean cortisol levels or the presence of cortisol pulses and SNAP-PE scores. The primary aim of the study was to describe pulsatility, and in order to be included in the study, an indwelling umbilical arterial catheter had to be in situ. This immediately selected a subgroup of infants who were possibly more unwell than those who had had their catheters removed and had BW < 1000 gr. Many of the infants were receiving dopamine, insulin, indomethacin etc, which implies that the infants were a 'sick' subgroup, and the mean SNAP-PE score was high (27). Therefore the relationship between cortisol levels and illness severity was not sought across the range of degrees of illness severity and may account for the lack of relationship demonstrated (Jett *et al.*, 1997).

Heckmann *et al* studied cortisol levels in preterm infants <30 weeks GA, some of whom were hypotensive and treated with catecholamines. SNAP-PE and NTISS scores were performed at birth and on the day of cortisol measurement. Of the ill, normotensive infants, 15/25 had infection associated with CRP > 10 mg dL<sup>-1</sup>, but the group did not have statistically higher cortisol than the controls. This is not in keeping with the possibility that very high cortisol levels may be due to bacterial infection. Hypotensive infants had a double peak of cortisol levels, higher and lower than controls. The incidence of proven or likely infection in the hypotensive infants with extremely high cortisol values (median 1200 nmol L<sup>-1</sup>) is not given, nor is an explanation offered for the extremely high values found in this sub-group. The authors found no difference in cortisol levels between those responsive and those unresponsive to hydrocortisone for the treatment of hypotension. Despite the finding of severity of illness scores being significantly different between the groups, controls < ill normotensive < ill hypotensive, no difference in illness severity was found between those with high and those with low cortisol levels and ill normotensive infants did not have statistically different cortisol levels from controls (Heckmann *et al.*, 2000).

#### **Studies finding a difference in cortisol between well and sick infants or a positive correlation between clinical features and cortisol**

Serum cortisol concentrations on day 14 were found to be significantly higher in infants requiring mechanical ventilation compared to non-ventilated infants ( $P = 0.014$ ) (Ng *et al.*, 1997c).

a: A ratio was found to correlate negatively with cortisol levels, 0.05,  $r = -0.3$ , in ventilated infants. This was not such a strong correlation as with plasma catecholamines (Barker and Rutter, 1996).

Reynolds found significantly higher cortisol in infants with fatal RDS both at 3 and 7 days when compared with non-fatal RDS affected infants. Both well and sick infants responded to ACTH. He concluded that an inadequate adrenal response did not contribute to the pathogenesis of RDS. He found no infants with 'inadequate' adrenal function (Reynolds, 1973).

92 neonates were investigated at 8:00 AM and PM on the second, fourth, and sixth days of life. Respiratory distress syndrome, especially in fatal cases, was found to be associated with an elevation in neonatal cortisol levels (Kaupila *et al.*, 1978).

In a study of 314 infants born at GA 24-32 weeks, the Clinical Risk Index for Babies (CRIB) was positively associated with cortisol level on days 1 and 3 through 7 (Banks *et al.*, 2001b).

Economou *et al* found that cortisol levels were higher in first 5 days than the rest of first month, with this difference more pronounced in the sick infants. They suggested that the higher cortisol levels ( $p <$



0.001) he and co-workers observed in sick preterm and full-term neonates, when compared to their respective controls, suggested an appropriate response to stress (Economou *et al.*, 1993).

The adrenocortical response to stress was studied longitudinally in 10 ill preterm infants using measurements of cortisol and 17 OH-P concentrations in filter paper blood spots. Mean cortisol and 17 OH-P concentrations reached a peak of 2200 nmol L<sup>-1</sup> and 65 nmol L<sup>-1</sup>, respectively, between the third and fifth days of life. These concentrations far exceeded those observed in older children and adults subjected to stress as a result of surgery. Further pulses of endogenous cortisol production of 4000 nmol L<sup>-1</sup> or more occurred in association with clinical complications such as intraventricular haemorrhage (Hughes *et al.*, 1987).

Sick infants >27 weeks GA increased their cortisol values from day 2 to day 6 although cortisol values decreased in well infants. In contrast, cortisol values significantly decreased from day 2 to day 6 in both well and ill infants that were ≤ 27 weeks GA (Scott and Watterberg, 1995).

**Clearly defined groups of infants, standardised tools for assessing illness severity and standardised postnatal ages and gestational ages may have facilitated the direct relationship becoming evident in these studies.**

#### **Studies finding an inverse relationship between cortisol and degree of illness**

Scott & Watterberg found lower cortisol values in infants that had the highest ventilatory requirements, that received surfactant or ionotropic support compared with values from infants without these requirements. Illness severity was assessed by RAS score, which is based on ventilatory respiratory illness (Scott and Watterberg, 1995). The reason that higher cortisol associated with more severe illness was not found in this study may be that only indicators of respiratory illness were used, rather than a more comprehensive score such as SNAP, that would include infective, biochemical and other sources of 'illness stress'. Also 'requirement' for interventions, such as ionotropes, surfactant, and ventilatory support are non-standard measures: there may be considerable intra and inter clinician or unit variation in threshold for intervention. It might also be that more infants of GA 24-27 weeks were included in the study, and these infants constitute a subgroup more likely to suffer from adrenal insufficiency.

In a study of 21 preterm infants, ventilated infants were found to have lower levels of baseline cortisol than non-ventilated infants. The ventilated infants had significantly higher SNAP scores than non-ventilated infants (Huysman *et al.*, 2000). This study found no relationship between SNAP scores and baseline cortisol, and a negative correlation between ACTH stimulated cortisol levels and SNAP scores. This second finding is in keeping with the fact that a blunted response to ACTH is observed in adult ITU patients who have high basal cortisol levels (Jurney *et al.*, 1987).



## 9.2 Appendix 2 Studies with total plasma cortisol values > 1000 nmol L<sup>-1</sup>

Economou describes 2 infants who subsequently died who had plasma cortisol levels consistently > 1600 nmol L<sup>-1</sup>. Mean levels are quoted, although the distribution is visibly skewed. Mean levels of preterm sick infants were 360 nmol L<sup>-1</sup>, and term sick infants were 268 nmol L<sup>-1</sup>, although no standardised description or gradation of degree of sickness was given. 13 levels from samples from 60 infants were >1000 nmol L<sup>-1</sup> (Economou *et al.*, 1993).

The adrenocortical response to stress was studied longitudinally in 10 ill preterm infants using measurements of cortisol and 17 OH-P concentrations in filter paper blood spots. Mean cortisol and 17 OH-P concentrations reached a peak of 2200 nmol L<sup>-1</sup> and 65 nmol L<sup>-1</sup>, respectively, between the third and fifth days of life. These concentrations far exceeded those observed in older children and adults subjected to stress as a result of surgery. Further pulses of endogenous cortisol production of 4000 nmol L<sup>-1</sup> or more occurred in association with clinical complications such as intraventricular haemorrhage (Hughes *et al.*, 1987).

Heckmann *et al* found 2 outlying levels of cortisol of 1233 nmol L<sup>-1</sup> and 2050 nmol L<sup>-1</sup> in well infants (Heckmann *et al.*, 1999).

Mantagos *et al* found one level of plasma cortisol as high as 1377 nmol L<sup>-1</sup> in a well term infant following venepuncture (Mantagos *et al.*, 1991).

Barker *et al* found a range of cortisol levels of 26-7799 nmol L<sup>-1</sup> in 47 ventilated preterm infants GA median 28 weeks, at median 15 hrs PNA (Barker and Rutter, 1996).

Jett *et al* found the upper end of the range for infants <28 weeks GA, 4-6 days PNA to be 1503 nmol L<sup>-1</sup> (Jett *et al.*, 1997).

Heckmann *et al* found the median plasma cortisol of a group of preterm hypotensive infants to be 1200 nmol L<sup>-1</sup> (range 764-1482 nmol L<sup>-1</sup>) (Heckmann *et al.*, 2000).

Thomas *et al* quotes levels in the 1000 nmol L<sup>-1</sup> s and Reynolds quotes some post ACTH cortisol values >1000 nmol L<sup>-1</sup> (Reynolds, 1973; Thomas *et al.*, 1986).

### 9.3 Appendix 3 Studies finding low total plasma cortisol levels in sick

#### infants

Thomas et al found 5 of 23 stressed preterm infants had undetectable basal cortisol levels. They did however produce adequate cortisol response to high dose ACTH (Thomas *et al.*, 1986).

The majority of ill infants of mean GA 26 weeks in first week of life were found to have total plasma cortisol  $<200 \text{ nmol L}^{-1}$  (Kari *et al.*, 1996).

Hingre suggested that considering the degree of stress and degree of illness, preterm ( $<30$  weeks GA) newborns had low basal cortisol levels (mean  $207 \text{ nmol L}^{-1}$ ), comparable to term infants, but considerably higher precursors (17 OH-P and 11-deoxycortisol). This suggested that 11  $\beta$  hydroxylase activity was reduced. 5 of 23 infants were also found to have a sub-optimal response to ACTH.

Economou identified 4 of 15 sick term infants with total plasma cortisol values  $90\text{--}190 \text{ nmol L}^{-1}$  and 6 of 15 sick preterm infants with values  $100\text{--}220 \text{ nmol L}^{-1}$  who were termed 'poor responders'. The definition was based on levels being less than 2 SD of mean of controls (Economou *et al.*, 1993).

In a study of 31 ELBW infants, GA  $<28$  weeks, in the first 8 days of life, five of the infants, having a mean SNAP-PE score of 41 (ie sick infants), had serum total cortisol levels  $\leq 83 \text{ nmol L}^{-1}$ . Five infants had levels  $<136 \text{ nmol L}^{-1}$ . Estimated mean serum free cortisol concentrations in these infants ( $21 \text{ nmol L}^{-1}$ ) were comparable to estimated free cortisol levels diagnostic of adrenal insufficiency in sick adult patients (Hanna *et al.*, 1997).

Lee et al found that half of infants studied ( $n=7$ ) had cortisol  $<140 \text{ nmol L}^{-1}$ , and maximum of  $410 \text{ nmol L}^{-1}$ , and suggested an enzyme level block to adequate stress levels of cortisol (Lee *et al.*, 1989).

In another study, 60% of infants had basal cortisol  $<138 \text{ nmol L}^{-1}$ , 30% had basal cortisol  $<83 \text{ nmol L}^{-1}$ . The authors suggested that delayed maturation of adrenal response may result in physiologically inadequate cortisol concentrations in stressed very low birth weight infants (Korte *et al.*, 1996).

At the end of the first week of life, infants who subsequently developed BPD and prolonged oxygen dependence had significantly lower cortisol secretion in response to ACTH than infants who recovered without BPD. There was no difference in basal cortisol levels between the groups. The authors speculated that these babies may be unable to secrete adequate amounts of cortisol in a setting of increased stress, leaving them vulnerable to continuing lung injury (Watterberg and Scott, 1995).

Scott and Watterberg suggested that some infants with severe illness, ie with higher requirements for ventilatory support and requirement of surfactant, had inappropriately low cortisol levels (Scott and Watterberg, 1995).

An ACTH test was performed on day 4 in 21 infants, GA 25.6–29.6 weeks. The SNAP score was used to measure illness severity. Patients with an adverse outcome had significantly lower baseline cortisol/17 OH-P ratios and 60-minute cortisol levels during ACTH testing. The authors suggest an insufficient adrenal response to stress in sick ventilated very preterm infants with GA  $<30$  weeks compared with non-ventilated less sick infants (Huysman *et al.*, 2000).

#### 9.4 Appendix 4 Studies suggesting reduced enzyme activity as cause for cortisol insufficiency in sick or preterm neonates.

17 OH-P, 11-deoxycortisol, and aldosterone values have been found to be higher in sick preterm infants than in healthy preterm infants. In one study, compared to healthy full-term infants, the premature infants had significantly higher 17-hydroxypregnenolone, 17 OH-P, and DHEAS concentrations. The authors suggest that elevated steroid precursors in preterm infants and low cortisol levels in stressed sick preterm infants may indicate a relative immaturity of adrenal enzyme activity (specifically, 11  $\beta$ -hydroxylase) and inadequate adrenal reserve for stress (Lee *et al.*, 1989). Korte also postulated that an elevated mean 11-deoxycortisol/cortisol ratio indicated that decreased 11  $\beta$ -hydroxylase activity may limit cortisol production in some infants (Korte *et al.*, 1996).

Hingre *et al.* have also found that the mean basal substrate/product ratio of 11-deoxycortisol has been found to be markedly elevated in preterm infants compared to that in full-term infants. These findings are consistent with decreased activity of 11  $\beta$ -hydroxylase in preterm infants born at < 30 weeks GA. Decreased 11  $\beta$ -hydroxylase activity appears to be more prominent than the deficiency of 3  $\beta$ -hydroxysteroid dehydrogenase that has been found in infants with lesser degrees of prematurity, suggesting that 11  $\beta$ -hydroxylase activity may be regulated during fetal development to increase during the latter part of gestation. ACTH appeared to induce an increase in the activity of 11  $\beta$ -hydroxylase activity. The authors suggest that despite generally reduced activity of this enzyme in preterm newborns, the rate-limiting step in the production of cortisol might well be 3  $\beta$ -hydroxysteroid dehydrogenase (Hingre *et al.*, 1994).

Fujitaka however argues that the findings of raised steroid precursors may be due to antibody cross reactivity and so falsely elevated levels (Fujitaka *et al.*, 1997). Using HPLC, he did not reproduce the findings.

In contrast, in a study using HPLC on spot plasma samples, 17 OH-P was detectable in 8 of 33 preterm infants < 32 weeks GA. Levels were undetectable by 22 days PNA. None of these infants had 'low' levels of cortisol or cortisone. 17 OH-P was not detected in infants > 32 weeks GA (Nomura, 1997). A similar pattern was found with 11-deoxycortisol, with detection in 11/33, disappearance by 21 days PNA, absence in infants > 32 weeks GA and no association with low cortisol or cortisone.

##### **Raised 17-OHP in sick and preterm infants**

Higher baseline levels of 17 OH-P in preterm sick infants compared to term infants have been found by several authors (al Saedi *et al.*, 1995; Murphy *et al.*, 1983; Wallace *et al.*, 1987). High levels of the precursor 17 OH-P appear to be produced in response to ACTH, but lower cortisol in sick compared with well ventilated preterm infants, suggesting an inability to convert the precursor to cortisol (Huysman *et al.*, 2000). These findings are in agreement with the earlier results published by Thomas *et al.* Basal plasma 17 OH-P concentrations were significantly increased in sick and well preterm infants compared to term infants and the peak plasma 17 OH-P response to ACTH was significantly higher in preterm ill infants (Thomas *et al.*, 1986). Murphy *et al.* also found mean plasma 17 OH-P values were appreciably higher in ill term and healthy preterm infants compared with healthy term infants, but the highest values were found in ill preterm infants. None of the infants had adrenal disease but some very ill infants had plasma 17 OH-P values approaching those seen in untreated infants with congenital adrenal hyperplasia (Murphy *et al.*, 1983).

## 9.5 Appendix 5 Studies with data on changes in cortisol levels with postnatal age

### Term infants

In an early (1978) study of 12 term healthy new-borns, delivered by SVD, with chromatography followed by specific RIA (Sippell *et al.*, 1978a), cortisol remained elevated in comparison with later infancy, with the exception of a marked "glucocorticoid dip" in cortisol and corticosterone levels between 2 and 12 hours after birth (Sippell *et al.*, 1978b).

Kojima found day one cortisol levels in (presumably) normal term infants to be significantly lower than cord levels. Cortisol then rose over the first 5 days of life before decreasing again towards 10 days PNA (Kojima *et al.*, 1981).

The absolute free cortisol values in full-term infants were highest immediately after birth then they fell to the lowest level observed between the third and fifth days of life (Rokicki *et al.*, 1990).

### Preterm infants

Several authors have described a decline in cortisol levels over the first 2 weeks of life (Metzger *et al.*, 1993; Kanarek *et al.*, 1988; Kauppila *et al.*, 1978).

Similarly, Economou found a general decrease in cortisol level over 15 days PNA. In preterm infants, cortisol levels were higher in first 5 days than the rest of the first month, with this difference more pronounced in the sick infants (Economou *et al.*, 1993).

ACTH stimulation tests were performed in 15 relatively well premature infants born at 28-34 weeks GA. The mean basal levels of cortisol were significantly higher at 5-10 days PNA than at 27-31 days PNA (Noguchi and Reynolds, 1978).

In a study of 314 infants, 24 to 32 weeks GA, mean cortisol was  $85.5 \text{ nmol L}^{-1}$  at birth, reached maximal levels at 24 hours PNA ( $535.2 \text{ nmol L}^{-1}$ ), and decreased to  $162.8 \text{ nmol L}^{-1}$  at 14 to 28 days PNA (Banks *et al.*, 2001a).

In infants born after 30 weeks GA, cortisol levels rose after birth and remain elevated for 24-48 hours (Kraiem *et al.*, 1985).

Cortisol values obtained in the first 3 days of life were found to be higher on day 2 than on days 3-7. Values on day 3-4 were similar to those on day 5-7 PNA (Watterberg *et al.*, 2000).

Scott and Watterberg found that the postnatal pattern in cortisol values depended on GA. Ill infants more than 27 weeks GA increased their cortisol values from day 2 to day 6 although cortisol values decreased in well infants. These patterns resulted in a non-significant change over time for these age groups. In contrast, cortisol values significantly decreased from day 2 to day 6 in both well and ill infants that were  $\leq 27$  weeks PNA (Scott and Watterberg, 1995).

### Long-term changes with postnatal age

There appears to be a fall in total cortisol levels over the first 2 months of life in preterm infants (Arnold *et al.*, 1997; Noguchi and Reynolds, 1978; Wittekind *et al.*, 1993).

A systematic decrease in the free cortisol value was observed in infants over 3 months in preterm and term infants (Rokicki *et al.*, 1990).

### Studies with no changes in cortisol with postnatal age demonstrated

In infants  $<9$  days old, Arnold *et al.* found that there was no association between mean cortisol over 6 hours and postnatal age (Arnold *et al.*, 1998).

In only 'mildly ill' infants, 38-94 days old, PNA had no effect on mean plasma levels (Metzger *et al.*, 1993).

Following a fall immediately after birth, levels in preterm infants 31-35 weeks GA appeared to be stable over the first week of life (Lee *et al.*, 1989).

Kraiem found a rapid fall in cortisol levels after the first few hours following birth, to levels that remained stable for samples taken at 3 and 10 days PNA. This was the pattern for both term and preterm (32 $\pm$  2 weeks GA) infants (Kraiem *et al.*, 1985).

Ng et al found no difference in response to hCRH in a group of 14 infants < 32 weeks GA tested on days 7 and 14 (Ng *et al.*, 1997b).

Watterberg, Scott, et al. found no difference in cortisol levels in preterm infants over the first week of life, following the first 48 hours (Watterberg *et al.*, 2000).

Bettendorf, Albers, et al found no significant relationship between salivary cortisol levels and postnatal age in preterm infants aged 15-39 days, who were well and stable (Bettendorf *et al.*, 1998).

## 9.6 Appendix 6 Studies with data on changes in cortisol levels with gestational age

### Studies describing 'lower gestation: higher cortisol'

In a group of well infants (clearly defined in paper) studied in the first 2 weeks of life, born at < 30 weeks GA, lower GA was associated with higher values (log(10) (Heckmann *et al.*, 1999).

In a group of infants GA 24-36 weeks, during the first week of life, Scott and Waterberg described an inverse relationship between GA and cortisol concentrations, with the least mature infants having the highest random cortisol values (Scott and Watterberg, 1995).

Higher levels of cortisol were found in preterm (32 weeks GA) compared to term infants, aged 4-31 days PNA, in a study of 'spot' plasma samples (Nomura, 1997).

In a study of total and free plasma cortisol, the absolute free cortisol values in premature newborns at 3-5 days of life exceeded the values observed in full-term subjects (Rokicki *et al.*, 1990).

Economou studied infants over the first month of life and found higher control cortisol levels in the preterm group (GA 33.5 weeks GA) than the term group. Sick preterm infants had higher cortisol levels than sick term infants (Economou *et al.*, 1993).

Ng *et al* studied total of 140 preterm (mean GA 29.6 weeks) and term infants. Blood samples were taken at 24 hours (day 1), and on day 4-5 of life. On day 4-5, but not day 1, preterm infants' mean cortisol levels were higher than term infants' (Ng *et al.*, 2000).

### No changes in cortisol with gestational age demonstrated

In a study of salivary cortisol on day 3 of life, Azcarate found no difference between healthy term and healthy preterm (mean BW 1420gr) infants' levels (Azcarate *et al.*, 1997).

Kraiem compared term to preterm infants (32+/- 2 weeks GA) up to 10 days old and found no difference in mean cortisol levels. No details of the degree of clinical compromise of the preterm infants was given in the paper (Kraiem *et al.*, 1985).

In a group of infants born at 25-29 weeks GA, sampled on day 4, there was no relationship between baseline cortisol and GA in a study relating cortisol to illness severity (Huysman *et al.*, 2000).

In infants born at 31-35 weeks GA, sampled at 2-5 days PNA, baseline cortisol was not found to be related to GA by Lee *et al* (Lee *et al.*, 1989).

There was no association between mean cortisol over 6 hours and GA in a group of infants born at 25-33 weeks GA (Arnold *et al.*, 1998).

Cortisol levels at PNA 1 week were not related to GA in infants born 24-31 weeks GA (Midgley *et al.*, 2001).

Prematurity was found not to affect neonatal cortisol levels over the first week of life (Kauppila *et al.*, 1978).

In a recent study of 314 infants, 24 to 32 weeks GA, levels during the first week were not found to be associated with gestational age, but levels on days 14-18 were inversely related to GA (Banks *et al.*, 2001a).

In a study of preterm well, preterm ill (GA > 30 weeks), and term ill infants, basal plasma 17 OH-P concentrations were significantly increased in both groups of preterm infants but there was no significant difference in basal plasma cortisol concentrations, between the groups although they were highest in preterm ill infants (Thomas *et al.*, 1986).

In infants born 24- 34 weeks GA, GA had no effect on mean plasma levels but the highest mean cortisol levels and the highest production rates were noted to be in the 2 most premature infants (Metzger *et al.*, 1993).

'Ill' preterm infants at 21 days PNA, mean GA 26 weeks were found to have similar cortisol levels to healthy term infants during the first week of life (Kari *et al.*, 1996).

Hingre et al studied 25 infants < 30 weeks GA who were moderately sick on day 4 and found levels similar to well term infants, but with raised steroid precursors (Hingre *et al.*, 1994).



## **9.7 Appendix 7 Studies with data on the effect of antenatal steroids on neonatal cortisol levels**

### **No or little difference found in neonatal cortisol levels after antenatal steroid therapy**

Ng et al have published 3 studies of the CRH test in various groups of infants. In a hCRH study of 61 infants < 32 weeks GA and < 1500g, on days 7 and 14 PNA, the baseline and post-stimulation plasma ACTH and cortisol did not differ significantly between infants whose mothers received no antenatal corticosteroids, and those whose mothers received 1-2 doses or > 2 doses (mean 7.2 doses) of antenatal dexamethasone (Ng *et al.*, 1997c).

Similarly, carrying out the CRH test prior to dexamethasone therapy at mean 28 days PNA, on infants <1500g, Ng found no difference between those who did and those who did not receive antenatal steroids (Ng *et al.*, 1997a).

The authors also examined the effect of multiple (8) courses of antenatal steroids on the preterm infant's adrenal response to hCRH. The absence of a significant difference in post-stimulation hormone concentrations between days 7 and 14, and the similarity of their hormone responses with those of older children and adults, suggested that no severe pituitary-adrenal suppression had occurred. Nonetheless, there was evidence of mild adrenal suppression in some of the treated infants (Ng *et al.*, 1999).

In a study of cortisol levels over the first week of life in 92 infants, after maternal dexamethasone therapy, neonatal cortisol concentration was found to be lower 30-60 minutes after delivery, but from the second day on it was at the same level as in infants without maternal therapy (Kauppila *et al.*, 1978).

In a study of well preterm infants, 28-34 weeks GA, 5-31 days PNA, there were 4 of 15 infants whose mothers had antenatal steroids. Their baseline and post ACTH stimulation values were similar to those not exposed to antenatal steroids (Noguchi and Reynolds, 1978).

A study of infants 25-28 weeks GA, comparing 8 infants treated with antenatal steroids with 18 who were not, found that the median amplitude of the cortisol secretory burst of the steroid-exposed infants was significantly less than that of the controls but there was no difference in the mean cortisol levels between the groups (Arnold *et al.*, 1998).

In a DBPCRT of 74 patients, the responsiveness of the adrenal cortex of the newborn infants, evaluated by a 2-hr ACTH test at the age of 24 hrs, did not differ between infants whose mothers had received either betamethasone or placebo (Teramo *et al.*, 1980). Two further studies have examined the cortisol response to ACTH in infants exposed and not exposed to antenatal steroids. Neither found a significant difference (Ohrlander *et al.*, 1977; Wilson *et al.*, 1988).

### **Early (<1<sup>st</sup> week) difference found in neonatal cortisol levels after antenatal steroid therapy**

In a study of 136 infants treated with antenatal steroid, compared with controls, umbilical cord levels of DHEAS and cortisol were significantly reduced within the first 24 hours after initial treatment and remained significantly lower than in control infants through 4 days after initial treatment (Parker, Jr. *et al.*, 1996).

In a study of 215 betamethasone-treated infants and 117 untreated infants of 26-36 weeks GA, cortisol levels were suppressed within 6 hours of betamethasone treatment, decreased to 45% of the concentration in untreated infants and returned to normal by 7 days PNA. The authors, however, concluded that the suppression of fetal cortisol did not interfere with the pituitary-adrenocortical response to stress after birth (Ballard *et al.*, 1980).

Wittekind studied 25 infants born at 24-33 weeks GA and found that plasma cortisol was significantly decreased 1 week after birth in infants whose mothers had received antenatal steroid, but at no other time in the 2 month study period (Wittekind *et al.*, 1993).

### **Sustained difference found in neonatal cortisol levels after antenatal steroid therapy**

In a study of 33 infants < 32 weeks GA, levels of cortisol and cortisone were markedly decreased in 4 of 16 infants whose mothers had received multiple courses of antenatal steroids. Low levels lasted for 3-4 weeks (Nomura, 1997).



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